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Research Article

Enhanced electrotransformation of the ethanologen *Zymomonas mobilis* ZM4 with plasmids

The *Zymomonas mobilis* ZM4 strain with excellent ethanol-producing capabilities was the first strain of *Z. mobilis*, which was sequenced. This strain is resistant to transformation, and no previous study has shown a detailed protocol for electrotransfer of ZM4 with foreign DNA. In this work, many electrical and biological parameters were selected and evaluated in order to optimize the electrotransformation of ZM4. First, improved transformation efficiencies of 11 896, 99, 96 and 5989 transformants/μg DNA were separately achieved with shuttle plasmid pZB21-mini (3082 bp), pZB21 (5930 bp), pZA22 (6994 bp) and broad-host-range vector pBBR1MCS-2 (5144 bp) all prepared from *Escherichia coli* JM110. The crucial factors affecting the transformation efficiency included the source of the plasmid (the best strain was ZM4), origin and size of the plasmids, growth phase of the cells (the most ideal phase was early log phase with OD_{600} of 0.3–0.4), the electric field strength (generally 11.75 kV/cm–13.25 kV/cm) and the recovery time (3–24 h). Further, based upon the optimal transformation protocol mentioned above for replicative plasmids in ZM4, (i) the electrotransformation by recombinant plasmid pBBR1MCS-2-P*gap-FLP* (6880 bp) was an immediate success with the transformation efficiency 10^2 transformants/μg DNA; (ii) the site-specific integration efficiencies (expressed in terms of "per μg of DNA") of 3–6 integrating transformants was obtained using the integrating plasmid pBR328-*ldh*R-*cml*-*ldh*L (7447 bp). This study will assist genetic and biotechnological research of ZM4 and other *Z. mobilis* strains by providing information about suitable vectors and a more universal and reliable procedure for introducing DNA into this strain.

Keywords: Electroporation / Integration / Plasmids / Transformation / *Zymomonas mobilis*

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1 Introduction

Zymomonas mobilis is a Gram-negative bacterium and an attractive and important ethanologen for cost-competitive ethanol production. *Z. mobilis* can also produce other high-value chemicals such as sorbitol, levan, or phenylacetylcarbinol and has attracted interest for its unusual membrane steroid content. Being regarded as a safe organism, *Z. mobilis* is even used for medicinal purposes, which further facilitates its utilization in large-scale biotechnological endeavors. The ZM4 is one of the most important strains of *Z. mobilis*, which was isolated from sugar cane juice and found to be a better ethanol producer than its parental strain CP4 or other strains [1–3]. This feature, in addition to its ability to be genetically manipulated, led to the sequencing of its genome, the results of which have provided insights into many of its characteristics [4, 5].

Unfortunately, the genetic manipulation of *Z. mobilis* is still restricted since all known *Z. mobilis* strains are inherently resistant to a variety of antibiotics [6, 7], most strains of *Z. mobilis* contain several indigenous plasmids [6, 7], and no bacteriophage has been reported for *Z. mobilis* yet [8]. More importantly, *Z. mobilis* has a strong host-dependent restriction/modification system and *rec*A-dependent repair system, causing it to be resistant to transformation and be highly

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Abbreviations: CFU, colony forming units; **Cm**, chloramphenicol; **Km**, kanamycin; **Tc**, tetracycline

resistant to mutagenesis [9]. However, these characteristics have still not been fully explained from analysis of the genomic sequence.

Among the diverse transformation protocols proposed for *Z. mobilis*, electroporation is the most convenient method and has been reported [10–19]. But in most of these papers, the electroporation protocols were only briefly described as part of the methods section and constituted minor points [13–19]. At the same time, ZM4 as the transformed host was seldom reported [15, 17]. A mutant strain with enhanced acetate resistance, ZM4/AcR, was transformed by electroporation with a plasmid encoding for xylose metabolism, pZB5 [15]. The plasmid pZB5 was extracted from ZM4 (pZB5) [19], and the transformation efficiency was found to be 2.6×10^3 transformants/μg DNA [15]. The broad host range vector pBBR1MCS-2 was further shown to be transferred into ZM4/AcR (pZB5) via electroporation with the transformation efficiency of 2×10^3 transformants/μg DNA [17].

Most of the above-discussed papers were related to transformation of *Z. mobilis* with replicative plasmids by electroporation [10–12, 14, 15, 17], with three papers related to chromosomal integration of *Z. mobilis* with integrating plasmids by electroporation [13, 16, 18]. These three papers have all reported *Z. mobilis* ATCC29191 as the host strain.

Although electroporation has been developed into a relatively mature genetic manipulation method for *Z. mobilis*, the current knowledge of how to transfer genes into this species is far from satisfactory for *Z. mobilis* research. First, the results described above were obtained from electroporation protocols optimized for a specific strain and/or a specific plasmid, which were difficult to generalize for other applications. Therefore, it was necessary for researchers to continuously re-evaluate and modify existing electroporation protocols for different strains and plasmids. Second, for some of the most important strains, including ZM4, data from electroporation protocols are still limited due to proprietary interests of commercial entities [15, 17].

In our preliminary experiments by original protocols based on the reported papers [10–12, 14, 15, 17], transformation of ZM4 with plasmids by electroporation was found to generally be more ineffective than CP4. In this work, in order to obtain a more universal and effective electrotransformation protocol of ZM4 by different kinds of plasmids, ZM4 was first transformed by several replicative plasmids, and the influences of many key electrical and biological parameters on the transformation efficiency were studied. The resulting optimal transformation protocol was further used for the transformation by a replicative recombinant plasmid and for chromosomal integration by a specific integrating plasmid to demonstrate whether this improved method can also work well with rare events.

2 Materials and methods

2.1 Bacterial strains, plasmid constructs and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. ZM4 was the transformation recipient. *Escherichia coli* strains Top10, JM110 and *Z. mobilis* CP4 were used for plasmid preparation and Top10 occasionally for the recovery of plasmids from ZM4 transformants. These strains were stored in 15% glycerol at −70°C.

In this work, six different plasmids were used. pZB21-mini, pZB21, pBBR1MCS-2-P*gap-FLP* and pBR328-*ldh*R-*cml*-*ldh*L were constructed in our lab (Fig. 1) [22, 25]. pZB21 was constructed from pBR328 and included a replicon region of the natural plasmid pZM2 from *Z. mobilis* ATCC10988 [22]. The *Bsp*T104I and *Nco*I restriction enzyme sites were introduced to the ends of the 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. The plasmid pBBR1MCS-2 is different from the three shuttle plasmids and has been proved to stably exist in many Gram-negative bacteria [24]. After digestion with *Cla*I and *Xba*I, the DNA fragment that contains the promoter region of the *Z. mobilis gap* gene (P*gap*) [26] and the *FLP* gene from the pCP20 plasmid [27] was ligated into the *Cla*I-*Xba*I sites of pBBR1MCS-2 generating pBBR1MCS-2-P*gap-FLP*.

Z. mobilis ZM4 and CP4 were statically grown overnight in rich medium (RM) medium (2% D-glucose, 1% yeast extract, 0.2% KH_2PO_4, pH 6.0) [10] at 30°C. Cultures on RM agar plates (1.8% agar) were incubated at 30°C for 2–4 days. Another growth medium that was used only in the original protocol was called TM (2% glucose, 1% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)_2SO_4$ and 0.2% KH_2PO_4) [15, 17]. The population sizes or the cell density (CD), which was expressed as CFU/mL, was determined by performing plate counts with appropriate dilutions of the culture. *E. coli* was grown in Luria Bertani (LB) medium (1% tryptone, 1% NaCl and 0.5% yeast extract), with shaking at 190 rpm at 37°C. Solid media were obtained by adding 1.8% w/v agar.

Because *Z. mobilis* strains are inherently resistant to a variety of antibiotics [6, 7], in preliminary experiments, the minimum inhibitory concentrations (MICs) toward tetracycline (Tc), kanamycin (Km) and chloramphenicol (Cm) in ZM4 were first identified and shown to be 10, 200 and 45 μg/mL, respectively. Then different Tc, Km and Cm concentrations higher than the MICs were tested and Tc 17.5 μg/mL, Km 310 μg/mL and Cm 100 μg/mL were showed to be suitable for genetic selections or plasmid maintenance in ZM4, which would allow ZM4 transformants to grow well and ZM4 not to grow. Tc 17.5 μg/mL was also shown to be suitable for CP4 by the same method. Hence, antibiotics were added at the following concentrations when needed: Tc 17.5 μg/mL for *Z. mobilis* and 15 μg/mL for *E. coli*; Km 310 μg/mL for ZM4 and 50 μg/mL for *E. coli*; Cm 100 μg/mL for ZM4 and 34 μg/mL for *E. coli*.

2.2 DNA procedures and PCR

Plasmid preparations, restriction enzyme digestions, ligations, *E. coli* transformations, DNA electrophoresis, plasmid concentration determination and Southern experiments were performed according to the standard protocols [28, 29]. It

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Essential properties	Source or reference
Z. mobilis ZM4(ATCC31821)	Wild strain, Genome-sequenced	[20]EMBL:AE008692
Z. mobilis CP4(NRRL B-14023)	Wild strain	[3]
Escherichia coli Top10	F$^-$ *mcr*A Δ(*mrr-hsd* RMS-*mcr*BC) ϕ80 *lacZ*ΔM15 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(*lac-pro*AB) [F′ *tra*D36 *pro*AB *lac*I ^{q} ZΔM15]	[21]
pZB21-mini	Shuttle vector, Tc^r, 3082bp	In our lab
pZB21	Shuttle vector, Ap^r, Cm^r, Tc^r, 5930bp	[22]
pZA22	Shuttle vector, Cm^r, Tc^r, 6994bp	[23]
pBBR1MCS-2	Broad-host-range plasmid, Km^r, 5144bp	[24]; GenBank: U02374
pBBR1MCS-2-P*gap-FLP*	pBBR1MCS-2 derivative, Km^r, 6880bp	In our lab
pBR328-*ldh*R-*cml*-*ldh*L	pBR328 derivative, integrating vector, Tc^r, *ldh*R[a)] (1483bp), FRT-Cm^r-FRT, *ldh*L(1419bp), 7447bp	[25]

a) *ldh*, gene encoding lactate dehydrogenase, used for homologous arms.

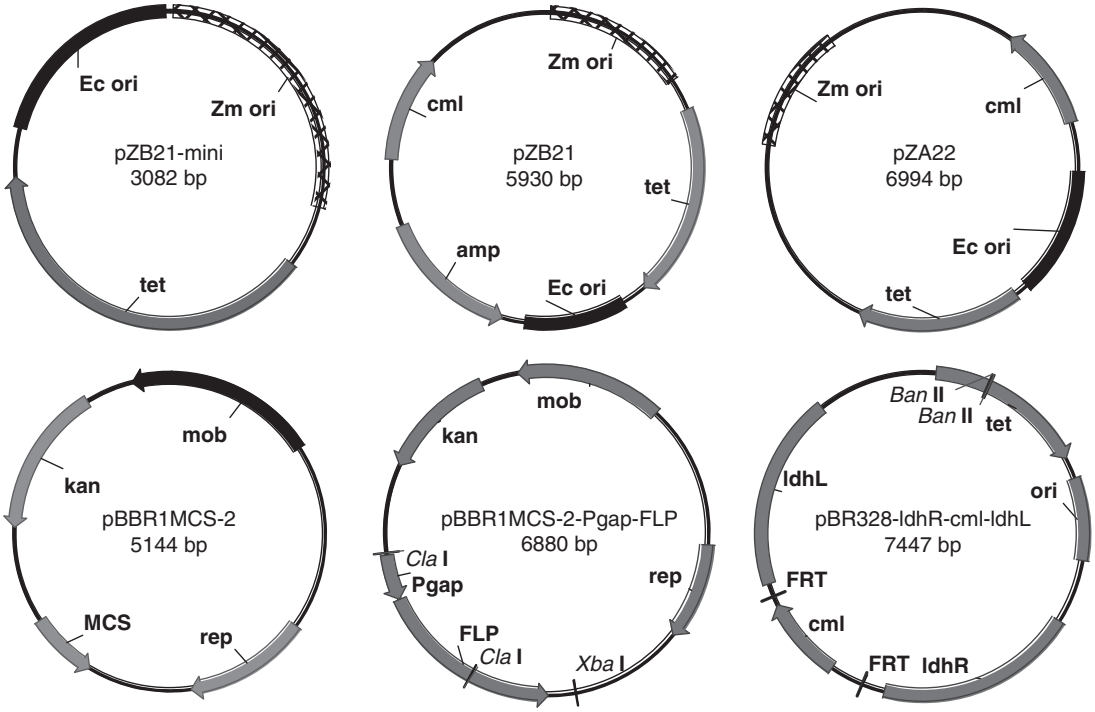

Figure 1. Diagram illustrating the six plasmids used in this study. *E. coli* origins of pZB21-mini and pZB21 were from pBR328, *E. coli* origin of pZA22 was from pACYC184, *Z. mobilis* origin was from pZM2; *tet*, tetracycline-resistance gene; *cml*, chloramphenicol-resistance gene; *amp*, ampicillin-resistance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

should be mentioned that all plasmids used for the transformation of ZM4 were prepared from the JM110 strain with the plasmid extract kit (Wizard Plus Midipreps DNA Purification system, Promega) unless otherwise indicated, and their concentrations were modified at the 125 ng/μL (pZB21-mini), 500 ng/μL (pZB21), 500 ng/μL (pZA22) and 150 ng/μL (pBBR1MCS-2), respectively. Genomic DNA was extracted as described before [25]. DIG High Prime DNA Labeling and Detection Starter Kit I was used (Roche).

DNA fragments were recovered from agarose gels using the Wizard SV Gel and PCR Clean-Up system (Promega) for extraction of the DNA. All colony PCR reactions were performed in a TGradient Thermocycler (Biometra, Germany) using Taq (Takara) and/or LA Taq (Takara) DNA polymerases.

2.3 Electroporation protocol

Cultures of *Z. mobilis* at early-log phase (optical density at 600 nm, $OD_{600} = 0.3–0.4$) and/or mid-log phase ($OD_{600} = 0.4–1.0$) were pelleted and washed in sterile ice-cold 10% glycerol, centrifuged then resuspended in 10% glycerol to yield final concentrations in the range of 10^9–10^{10} cells/mL. The final cell concentration was determined quickly by using the Petroff-Hausser Counter, followed by plate counts with appropriate dilutions of the culture, if necessary. Plasmid DNA (dissolved in sterile water) was added to 40–200 μL aliquots of the cells. The plasmid/cell suspension was transferred to chilled 2 mm-gap cuvettes (Bio-Rad) and kept on ice for 2 min, then placed between electrode plates of the electropulser Micropulser (Bio-Rad), and an appropriate pulse was applied. The capacitance and resistance of the Micropulser were kept constant and set at 25 μF and 200 Ω. An aliquot of the suspension was mixed with 0.8 mL of prewarmed recovery medium (RM was used unless otherwise indicated) and kept for 3–28 h at 30°C. A sample would be taken from the mixture before its incubation at 30°C for testing the cell density by plate counts, if necessary. 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 48–96 h at 30°C were replicated with toothpicks 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 [28, 29]. For pBR328-*ldh*R-*cml*-*ldh*L, the colonies growing on selective agar plates (RM+Cm) were identified by successive plating onto RM+Tc, RM+Cm and RM agar plates, and then characterized by PCR using genomic DNA as template [25] and Southern hybridization [28, 29]. Transformation efficiency was calculated as the number of Tc-, Km- or Cm-resistant transformants per μg of plasmid DNA added. The control samples without DNA and the study samples underwent the same protocol, except that the study samples also underwent electroporation.

The post-pulse "recovery" media evaluated were: RM, SOC (20 mM glucose, 2.5 mM KC1, 10 mM $MgC1_2$, 10 mM $MgSO_4$, 10 mM NaCl, 2% tryptone and 0.5% yeast extract), modified rich medium (MRM) (27.8 mM D-glucose, 18.9 mM $(NH_4)_2SO_4$, 1.47 mM KH_2PO_4 and 1 mM $MgSO_4 \cdot 7H_2O$, 0.5% tryptone, 1% yeast extract).

3 Results

In preliminary experiments by original protocols based on the reported papers [10–12, 14, 15, 17], the transformation of ZM4 with pZB21-mini, pZB21 and pZA22, and pBBR1MCS-2 by electroporation was all successful, showing that those plasmids could replicate stably in *Z. mobilis*. At the same time, their transformation efficiencies were found to differ greatly from each other. Further, their derivative plasmids with increasing size were often difficult to be introduced into ZM4. Thus, it is necessary to further optimize the transformation method. All data were from two or three independent experiments.

In the light of unavoidable variations in transformation efficiency, plasmids with very low transformation efficiency were considered inappropriate for testing of the parameters. Further, because pZB21-mini, pZB21 and pZA22 have the same replication origin in *Z. mobilis*, so pZB21-mini was first selected for evaluating and optimizing electroporation parameters in this study.

3.1 Electrotransformation of ZM4 by pZB21-mini

Nine parameters were independently evaluated and optimized. One or more of those parameters have not or have seldom been reported before, perhaps due to other researchers' view of their minor influence on transformation efficiency. However, in our study, we systematically tested these parameters in the transformation of ZM4 and found that, in fact they have been proven not to be negligible at all and helped to improve the transformation efficiencies when optimized, as we would see later in the text.

The first was the cuvette and the volume of competent cells in the cuvette. The cuvettes with a 1-mm-gap width and with a 2-mm-gap width have been most widely used and their filling volumes have been often used with 40–120 μL and 40–200 μL, respectively. In our preliminary experiments, we found both of them could be worked well and the superiority by the use of the 1-mm-gap cuvette is the higher electric field strength could be obtained, but the disadvantage is the amount of the competent cells and DNA added would be more limited and the transformation efficiency would be more instable. Considering that the electric field strength obtained by the 2-mm-gap cuvette and the Micropulser (Bio-Rad) was probably adequate for the transformation of ZM4, we selected the 2-mm-gap cuvette in order to obtain the more universal, reliable and reproducible results.

As shown in Fig. 2, the optimal volume of competent cells for pZB21-mini was 120 μL with a transformation efficiency of 4488 ± 300 transformants/μg DNA. Thus, the 120 μL was immediately applied in all subsequent experiments.

The experiments for electric field strength test were simultaneously performed with the competent cell volume. As shown in Fig. 3, the optimal electric field strength was 13.25 kV/cm for pZB21-mini. The corresponding transformation efficiency was 4660 ± 341 transformants/μg DNA. We also found that the electroporation with the electric field strength of more than 14 kV/cm was apt to cause a number of complications, such as arcing and shortening of the electrical pulses, as reported by Szostková and Horáková [30].

As shown in Fig. 4, in an electroporated aliquot (1.03–5.15 ng/μL), an approximately linear relationship existed between the number of electrotransformants recovered and the DNA concentration and an approximately reverse relationship existed between the transformation efficiency and the DNA concentration.

The cell density in a cuvette is an important parameter for efficiency of electroporation. Clearly, the cell density of 1.77×10^{10}, 2.36×10^{10} and 2.95×10^{10} CFU/mL cell all achieved high transformation efficiency, and 2.36×10^{10} CFU/mL cell (corresponding to 100 × of the harvested culture

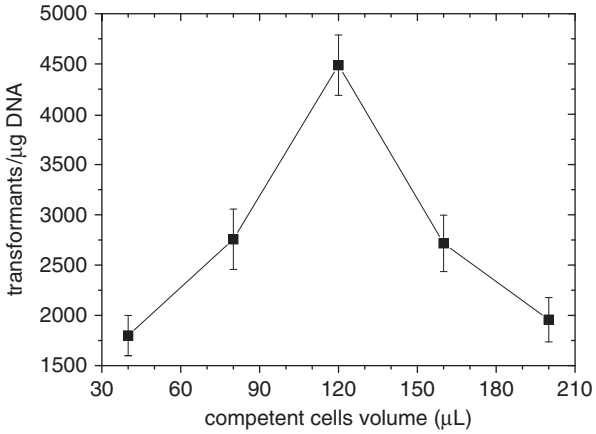

Figure 2. Effect of the volume of competent cell suspension in a cuvette on the transformation efficiency of ZM4 with pZB21-mini. ZM4 cultures $OD_{600} = 0.42$ was centrifuged, washed then resuspended in 10% glycerol at 1/100th of the culture volume (expressed as 100×). Freshly prepared competent cells were divided into 40, 80, 120, 160, 200 μL aliquots, and 1, 2, 3, 4, or 5 μL pZB21-mini added, respectively. The field strength, 13.25 kV/cm pulses; the RC time constant, 5.3–5.9 ms; recovery time, 23–24 h.

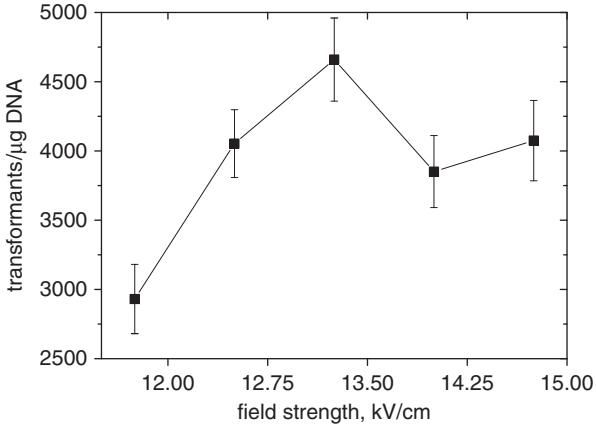

Figure 3. Effect of the electric field strength on the transformation efficiency of ZM4 with pZB21-mini. ZM4 cultures $OD_{600} =$ 0.42, 100×; 3 μL pZB21-mini was added. The RC time constant, 4.7–5.8 ms; recovery time, 23–24 h.

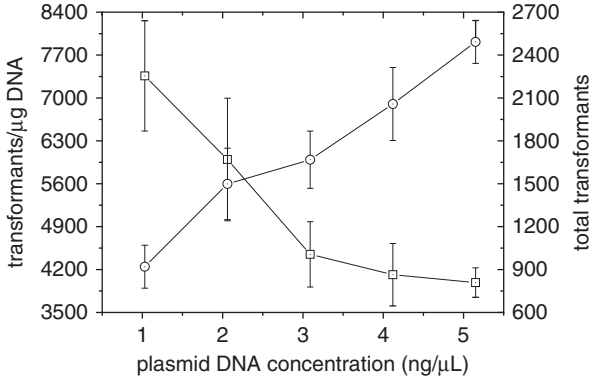

Figure 4. Effect of the plasmid concentration on the transformation efficiency of ZM4 with pZB21-mini. (□) transformants/μg DNA; (○) total transformants.ZM4 cultures ($OD_{600} = 0.51$), 100×(ibid); 1, 2, 3, 4 or 5 μL pZB21-mini added, respectively. The field strength, 13.25 kV/cm pulses; the RC time constant, 4.9–5.2 ms; recovery time, 23–24 h.

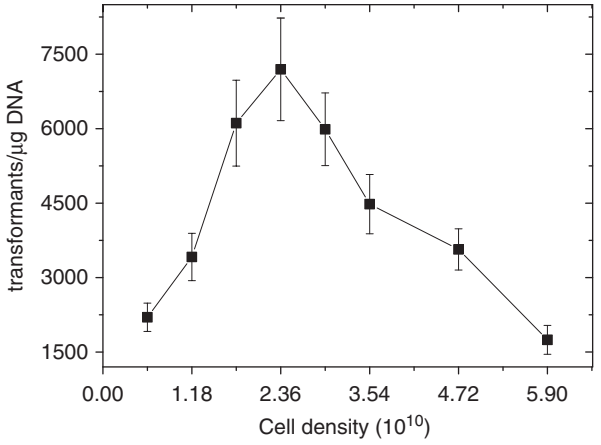

Figure 5. Effect of the ZM4 cells density on the transformation efficiency of ZM4 with pZB21-mini. ZM4 cultures $OD_{600} = 0.51$, $CD = 2.36 \times 10^8$; 100×. Freshly prepared competent cells were divided into 30, 60, 90, 120, 150, 180, 240, 300 μL aliquots, then all aliquots finally became 120 μL by adding additional 10% glycerol or removing partial supernatant fluid after centrifuging. 1 μL pZB21-mini added; the field strength, 13.25 kV/cm; the RC time constant, 5.0–5.9 ms; recovery time, 23 h.

volume) gave the highest 7196 ± 1034 transformants/μg DNA with the total of 899 ± 129 transformants (Fig. 5). It should be mentioned that different cell densities were obtained after additionally diluting or centrifugating the samples with different volumes of 100× of the harvested culture and it seemed that this treatment did not significantly affect the competence of the cells and the transformation efficiency (data not shown).

As for the source of plasmid DNA, four host strains, namely, Top10, JM110, CP4 (NRRL B-14023) and ZM4 were selected for obtaining pZB21-mini because of the differences in their genetic backgrounds (Table 1). The condition was: ZM4 cultures $OD_{600} = 0.645$, 100×, the field strength 13.25 kV/cm and the recovery time 17.5–18 h. ZM4 was shown to be the best host for the plasmid, giving the highest transformation efficiency with $(6.13 \pm 1.16) \times 10^4$ transformants/μg DNA. The transformation efficiencies for Top10, JM110 and CP4 were $(1.28 \pm 0.13) \times 10^2$, $(2.50 \pm 0.26) \times 10^3$ and $(3.60 \pm 1.03) \times 10^3$ transformants/μg DNA, respectively.

The recovery time to obtain maximum number of total transformants was also evaluated. As shown in Fig. 6, the transformation efficiency of pZB21-mini was high at 3, 11 or 16 h, but it was the highest at 11 h, with up to total 965 total transformants and 7720 ± 1544 transformants/μg DNA.

Generally, freshly prepared competent cells are recommended to be used with some exceptions [31]. In order to

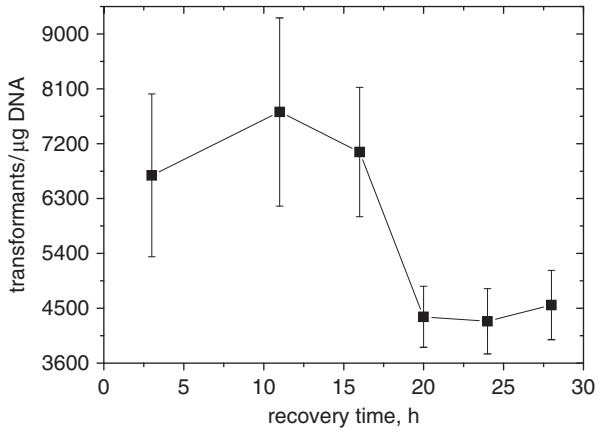

Figure 6. Effect of the recovery time of transformed cells on the transformation efficiency of ZM4 with pZB21-mini. ZM4 cultures $OD_{600} = 0.594$, 100×; 1 μL pZB21-mini was added. The field strength, 13.25 kV/cm; the RC time constant, 4.9–5.2 ms. An aliquot of the suspension was plated at different recovery times.

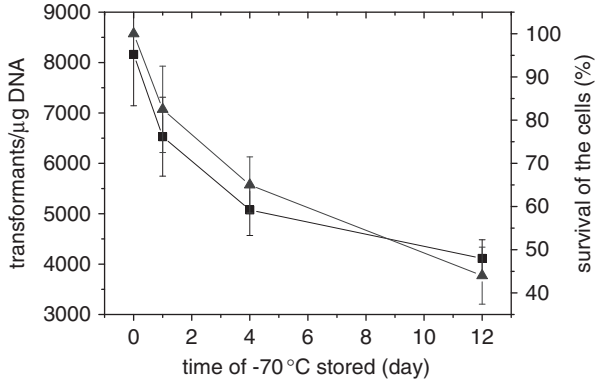

Figure 7. Effect of the time of −70°C stored on the survival of the cells and the transformation efficiency of ZM4 with pZB21-mini. (■) transformants/μg DNA; (▲) survival of the cells. ZM4 cultures $OD_{600} = 0.560$, 100×. Freshly prepared competent cells were divided into 120 μL aliquots and some of them were stored in −70°C freezer for 1–12 days. 1 μL pZB21-mini added. The field strength, 13.25 kV/cm; the RC time constant, 4.8–5.7 ms; recovery time, 11 h.

determine the true effect of cells frozen at −70°C on transformation efficiency, different times of cells stored at −70°C were tested. As shown in Fig. 7, storage at −70°C had a negative effect on transformation, regardless of time. The proportionally decreasing survival of the cells maybe the main cause for the negative effect (Fig. 7).

The growth phase of bacterial cells was another important parameter influencing the transformation efficiency. Under the conditions of 1 μL pZB21-mini, the 13.25 kV/cm field strength and the 11 h recovery time, early-log growth phase ($OD_{600} = 0.375$) resulted in the optimal transformation efficiency with $(1.18 \pm 0.32) \times 10^4$ transformants/μg DNA, in agreement with the report by Okamoto and Nakamura [10]. The transformation efficiencies were $(8.41 \pm 1.24) \times 10^3$ and $(4.37 \pm 0.48) \times 10^3$ transformants/μg DNA for $OD_{600} = 0.570$ and 0.903 cultures, respectively.

Our experimental results further showed that the transformation efficiency $(1.92 \pm 0.40) \times 10^3$ transformants/μg DNA with SOC as recovery medium was far lower than that of $(9.74 \pm 2.80) \times 10^3$ transformants/μg DNA with RM, as reported also by Lam et al. [11], but the transformation efficiency $(6.90 \pm 1.41) \times 10^3$ transformants/μg DNA with MRM was not higher than with RM, unlike that reported by Jeon et al. [15, 17]. The conditions were same as the above except ZM4 cultures $OD_{600} = 0.428$.

3.2 Initially optimized transformation protocol by electroporation

In view of the results obtained from nine parameters test and evaluation with pZB21-mini, initially optimized transformation protocol for ZM4 was proposed as the followings: ZM4 early-log growth phase ($OD_{600} = 0.3$–0.4) cultures were centrifuged, washed then resuspended in 10% glycerol at 1/100th of culture volume (100×) to yield final 10^{10} cells/mL concentrations. Freshly prepared competent cells were divided into 120 μL aliquots and immediately used. Generally, no more than 3 μL of plasmid extracts from JM110 was added. The 2-mm-gap cuvette was selected. The field strength was 13.25 kV/cm, and 0.8 mL of preheated RM was used as recovery medium with a recovery time of 11 h at 30°C.

3.3 Electrotransformation of ZM4 by pZB21, pZA22 and pBBR1MCS-2

Above protocol was further evaluated and optimized with pZB21, pZA22 and pBBR1MCS-2. We found that initial conditions with these seven parameters except electric field strength and recovery time for pZB21-mini transformation were completely suitable for the three plasmids tested. The exceptions were the optimal field strength was 11.75 kV/cm for pZA22 and pBBR1MCS-2 (Fig. 8), differing from that of 13.25 kV/cm for pZB21-mini (Fig. 3). The corresponding transformation efficiency was 12 ± 4 and 2424 ± 273 transformants/μg DNA, respectively. pZB21 was transformed into ZM4 with a slightly higher efficiency (18 ± 5 transformants/μg DNA) than pZA22 under similar conditions (ZM4 $OD_{600} = 0.42$, field strength 11.75 kV/cm). Thus, the optimal field strength varied with plasmids and decreased with increasing size of the plasmids, as reported by Szostková and Horáková [30].

Under the field strength of 11.75 kV/cm, the optimal results of recovery time of transformed cells for pZA22 and pBBR1MCS-2 are shown in Fig. 9. The transformation efficiency of pZA22 was highest at 11 h, with up to 60 ± 20 transformants/μg DNA. However, the optimal recovery time for pBBR1MCS-2 was 24 h, and the transformation efficiency was 2604 ± 651 transformants/μg DNA. pZB21 was transformed into ZM4 with an efficiency of 68 ± 16 transformants/μg DNA under the conditions similar to that with pZA22 (ZM4 $OD_{600} = 0.375$, field strength 11.75 kV/cm, recovery time 11 h). Thus, the recovery time to obtain the maximum

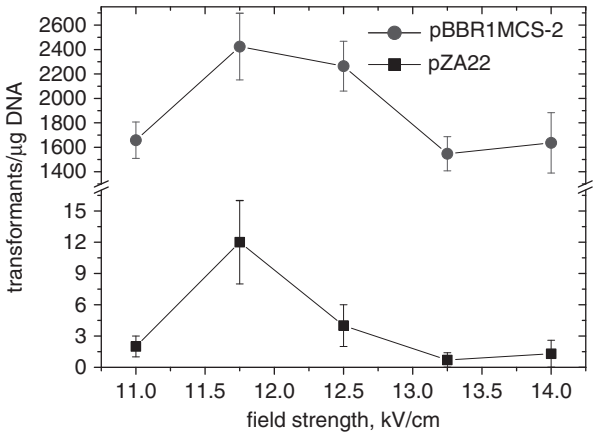

Figure 8. Effect of the electric field strength on the transformation efficiency of ZM4 with pZA22 and pBBR1MCS-2. ZM4 cultures $OD_{600} = 0.42$; 3 μL pZA22 and pBBR1MCS-2 added, respectively. The RC time constant, 5.4 ms for pZA22, 5.0–5.5 ms for pBBR1MCS-2; recovery time, 23–24 h.

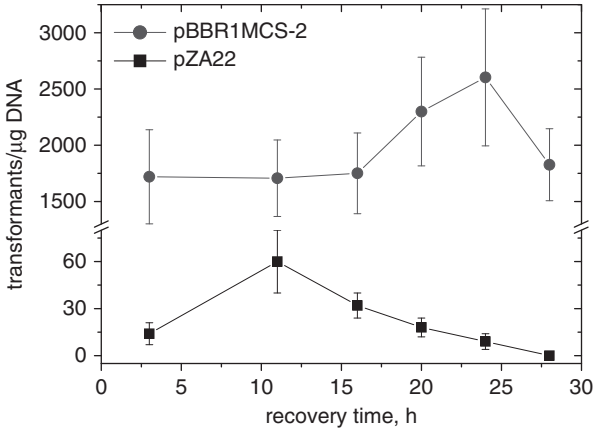

Figure 9. Effect of the recovery time of transformed cells on the transformation efficiency of ZM4 with pZA22 and pBBR1MCS-2. ZM4 cultures $OD_{600} = 0.375$; 3 μL pZA22 and pBBR1MCS-2 added, respectively. The field strength, 11.75 kV/cm; the RC time constant, 4.9–5.2 ms. An aliquot of the suspension was plated at different recovery times.

number of total transformants and the resultant maximum transformation efficiency also varied with plasmids.

Because of relatively low transformation efficiency of ZM4 by pZB21 or pZA22, the phage protein "ocr" [32], a TypeOne Restriction Inhibitor (EPICENTRE Biotechnologies, USA) was attempted to use according to manufacturer's instructions. TypeOne Restriction Inhibitor can be electroporated into cells along with transforming DNA and increases the transformation efficiencies of unmodified DNA in bacterial strains with Type I R-M systems by in vivo blocking the DNA binding site of Type I R-M enzymes and inhibiting cleavage of unmodified DNA [32]. Under the optimal parameters as mentioned above and with 2.5 μg of TypeOne Restriction Inhibitor added, the transformation efficiencies of ZM4 by pZB21, pZA22, pBBR1MCS-2 were 99 ± 24, 96 ± 22 and 5989 ± 1121 transformants/μg DNA, all higher than the controls without using TypeOne Restriction Inhibitor, 63 ± 16, 58 ± 14 and 2580 ± 510 transformants/μg DNA, respectively. TypeOne Restriction Inhibitor has been reported to be used in *Z. mobilis* transformation and the transformation efficiency of ZM4/AcR (pZB5) by pBBR1MCS-2 was 2×10^3 transformants/μg DNA [17].

3.4 Optimized transformation protocol by electroporation

In view of the results obtained from the further evaluation with pZB21, pZA22 and pBBR1MCS-2, optimized transformation protocol for ZM4 was proposed as the following: the field strength and the recovery time at 30°C would vary with the kind of DNA transformed. Their ranges are generally 11.75–13.25 kV/cm and 3–24 h, respectively. If necessary, TypeOne Restriction Inhibitor would be used to increase the transformation efficiency. The others are the same as before.

In order to check and apply the results that were mentioned above from the replicative plasmids encoding drug-resistant markers, electroporation of ZM4 by the non-replicative integrating plasmid pBR328-*ldh*R-*cml*-*ldh*L and of the resultant *ldh*A-deficient ZM4(*ldh*A::FRT-*cml*-FRT) strain by the recombinant pBBR1MCS-2-P*gap*-*FLP* carrying with the non-drug-resistant marker-encoding gene was further investigated. The results were shown in Sections 3.5 and 3.6.

3.5 Electrotransformation of ZM4 by pBR328-*ldh*R-*cml*-*ldh*L

For industrial applications, having special requirements for plasmid maintenance are undesirable. Thus, integration of the desired gene into the bacterial genome that would provide genetically engineered strains would be more acceptable for production purposes. However, such rare events in organisms as gene insertions and gene disruptions, generally require relatively high transformation efficiencies if electroporation is used. The final protocol was further used for chromosomal integration with the specific integrating plasmid pBR328-*ldh*R-*cml*-*ldh*L, which was constructed for disrupting the putative lactate dehydrogenase-encoding gene *ldh*A in ZM4 to reduce the lactate byproduct by electroporation [25]. It was shown that all transformants obtained were all resistant to Cm and sensitive to Tc. The identifying results by PCR method [25] further proved that all transformants were chromosomally integrated by double crossover events. The optimal result shown in Table 2 demonstrated that the field strength is still one of the most important parameters, and the optimized method could thus work well with rare events. In addition, linear vector was shown to be more efficient, which was confirmed repeatedly in at least four following independent studies on the disruption of other genes in ZM4 (unpublished data). The evaluation results of resultant ZM4 (*ldh*A::FRT-*cml*-FRT) strains showed that their lactate production decreased by 30–50% and their ethanol yields (g/g glucose consumed) slightly increased by 1.5–5% compared to ZM4, when cultured in RM media with 6% glucose as carbon source. Further investigation is under way.

Table 2. Site-specific integration efficiency of ZM4 with pBR328-*ldh*R-*cml*-*ldh*L from JM110[a)]

Samples	1	2	3	4	5	6	7	8
pBR328-*ldh*R-*cml*-*ldh*L	Circular plasmids, 1 μg/μL				*Ban*II-digested linear fragments, 1 μg/μL			
Electric field strength (kV/cm)	11.75	12.5	13.25	14.0	11.75	12.5	13.25	14.0
Total transformants	0	7±2	9±1	1±1	0	8±2	18±5	3±1
Transformants/μg DNA	0	2.33	3	0.33	0	2.67	6	1

a) ZM4 cultures $OD_{600} = 0.402$; 3 μL DNA added; recovery time, 15 h.

3.6 Electrotransformation of the *ldh*A-deficient ZM4 by pBBR1MCS-2-P*gap-FLP*

In order to attempt to use the FLP-FRT site-specific recombination system from *Saccharomyces cerevisiae* in *Z. mobilis* [33], pBBR1MCS-2-P*gap-FLP* was introduced into ZM4 (*ldh*A::FRT-*cml*-FRT) strain by electroporation, in which the FRT-*cml*-FRT fragment had been inserted into the ZM4 genome at the site of the putative *ldh*A gene [25]. The conditions were host cell cultures $OD_{600} = 0.397$, DNA concentration 300ng/μL, 3 μL DNA added, the field strength 11.75 kV/cm and the recovery time 15 h. The electrotransformation was an immediate success with the transformation efficiency 10^2 transformants/μg DNA, again indicating the effect of the optimized transformation protocol and of the size of plasmids on the transformation efficiency in ZM4 or ZM4-derivative strains. Further characterization of the transformants proved that all colonies on initial selective agar plates (RM+Km310) containing pBBR1MCS-2-P*gap-FLP* were also Cm-sensitive and deficient in *cml*, i.e. they were already ZM4 (*ldh*A::FRT) (pBBR1MCS-2-P*gap-FLP*) strains. In addition, pBBR1MCS-2-P*gap-FLP* could be conveniently removed to obtain ZM4 (*ldh*A::FRT) strains by serially transferring the cells several times into antibiotic-free medium.

4 Discussion

All the transformation efficiency data in this study has clearly showed that among the parameters tested in the transformation of ZM4, the replication origin and size of plasmids, source of plasmids, cell growth phase, electrical field strength and recovery time were shown to be more crucial factors than others to increase the transformation efficiency. While each factor can be independently assessed, their relationships with each other are complicated. It seems that interaction of the specific genetic background of the host and the specific genetic characteristics of the plasmid is the most important, while the physical electrical parameters are minor for the transformation efficiencies of different plasmids.

pZB21-mini, pZB21, pZA22, pBBR1MCS-2 and pBBR1 MCS-2-P*gap-FLP* vary with replication origin and size, and thus their transformation efficiencies in ZM4 greatly differed from each other. pZB21-mini (3082 bp), pZB21 (5930 bp) and pZA22 (6994 bp) has the same replication origin in *Z. mobilis*, and so their optimal transformation efficiencies in ZM4 were directly related to their size: 11 896, 99 and 96 transformants/μg DNA (from JM110), respectively. In spite of being 5144 bp in size, the transformation efficiency of pBBR1MCS-2 was far higher than those of pZB21 and pZA22 (also higher than that of 6880 bp pBBR1MCS-2-P*gap-FLP*, 10^2 transformants/μg DNA), but lower than that of pZB21-mini, with up to 5989 transformants/μg DNA (from JM110). The role of the size of the plasmids for the efficiency of the electrotransformation has already been described, and it is known that the number of transformants decreases as the size of the plasmid increases [30, 34–36]. However, the transformation efficiency of certain bacteria is not significantly affected by plasmid size [30]. Our work confirmed that the transformation efficiency of ZM4 is indeed significantly affected by plasmid size as well as the effect of the plasmid origin. In addition, it seems that the optimal electric field strength and recovery time are closely related to the origin type, size and conformation of plasmids (Fig. 8 and 9, Table 2).

In fact, the fundamental cause of the discussed above difference on transformation efficiencies of plasmids in ZM4 may mainly lie in the restriction-modification (R-M) systems in the organism. It is a general belief that transformation difficulties are mainly caused by the presence of R-M systems in the organism rather than physical factors involved in transformation procedures [37]. Our results seem to support this belief because that the source of bacterial plasmid DNA and the TypeOne Restriction Inhibitor could greatly influence the transformation efficiencies of plasmids. The transformation efficiency of ZM4 by pZB21-mini or pBBR1MCS-2 extracted from ZM4 was 478 or 268 times higher, respectively, than that of ZM4 by the same plasmid extracted from Top10; and 24.5 or 6.8 times higher, respectively, than that of ZM4 by the same plasmid extracted from JM110 (data for pBBR1MCS-2 not shown). This implies that ZM4 has a strict R-M system for exogenous DNA, and this system may be methyl-dependent, only cleaving DNA with methylated DNA, since the plasmids from JM110 would not be adenine- and cytosine-methylated DNA. According to the sequence of the ZM4 genome, it is predicted that to have Mrr, a Type IV system restriction protein, and its DNA sequence was identified to be more than 97% homologous with the *mrr* gene from CP4. The *mrr* gene of CP4 encodes a methyl-dependent restriction endonuclease ZmCP4Mrr [38]. Similarly, the source of bacterial plasmid DNA significantly influences the transformation efficiency of CP4 (data not shown). It was also predicted that type I R-M enzymes exist in ZM4. Our results with the use of the TypeOne Restriction Inhibitor supported the presence of Type I R-M system(s) in ZM4.

Further the difference on transformation efficiencies of plasmids, especially pZB21-mini, pZB21 and pZA22, is most likely to be relevant to RM system's restriction in ZM4 against the additional DNA fragment of pZB21 and pZA22, in contrast

Practical Application

Z. mobilis is an important ethanologen for cost-competitive ethanol production and can also produce other high-value chemicals such as sorbitol, levan, or phenylacetylcarbinol and has created interest because of its unusual membrane steroid content. There are no previous in depth studies on electrotransfer of foreign DNA for the ZM4 strain, which is the first fully sequenced strain of *Z. mobilis*, and which was used for industrial conversion of lignocellulose to ethanol by Dupont/Broin. The presented results will assist genetic and biotechnological research of ZM4 and other *Z. mobilis* strains. In addition, it will be beneficial for genetic engineering of other microorganisms by providing a detailed transformation protocol.

to pZB21-mini. Because the transformation efficiencies of pZB21 and pZA22 by using TypeOne Restriction Inhibitor were still relatively low, and Type II R-M systems are the most common of all R-M systems [38], this RM system is predicted to belong to Type II systems. It is regretful that the valuable clues for it are still inadequate (http://rebase.neb.com) [4, 38, 39].

Therefore, it is necessary for us to further understand the R-M system(s) in ZM4 and accordingly to further improve the genetic manipulation of ZM4. On the other hand, one of the important methods for identifying R-M system genes includes such rare events as gene insertions and gene disruptions. However, gene insertions or gene disruptions by homologous recombination generally need to be manipulated with integrating vectors by electroporation. Because of the very small probability of occurrence of such rare events, a significantly higher transformation efficiency with integrating vectors than with replicative plasmids is needed.

In conclusion, based on current knowledge and technology for genetic manipulation in ZM4, we systematically studied the electrotransformation of ZM4 and key factors affecting the efficiency of this process. We were able to obtain reliable and reproducible results, and the resulting optimal transformation protocol could work well with rare events. Obtaining stable transformation and integration of ZM4 represent an important step toward successful functional genomics studies of *Z. mobilis*. The complete genome sequence coupled with transformation systems will enable us to better exploit the biotechnological properties of this important industrial bacterium. Furthermore, the ability to feasibly manipulate the genes of ZM4 strain will allow improved production of ethanol fuel and higher value products.

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