ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Design and Construction of Improved New Vectors for *Zymomonas mobilis* Recombinants

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ABSTRACT: *Zymomonas mobilis* is a very important gram-negative bacterium having a potential application to simultaneous co-production of biofuel and other high value-added products through biorefinery process technology development. Up to now, pLOI193 has been used as the plasmid of choice for *Z. mobilis* strains. However, its application has been limited due to its relatively low transformation efficiency, a large plasmid size (13.4 kb), and limited choice of cloning sites for gene manipulations. Some of these limitations can be overcome by the newly designed and constructed plasmid pHW20a, which provides significantly higher transformation efficiency (about two orders of magnitude greater), better stability (for at least 120 generation times), and an ease of gene manipulations. The pHW20a contains three complete *cis*-acting genes (*rep*A, *rep*B, and *rep*C) encoding the Rep proteins for primosome formation. It has the origin of replication (*ori*V) to ensure replication in gram-negative bacteria, two *mob* genes that enhances transformation efficiency, a screening marker (*lacZα*), expanded multiple cloning sites (MCS) that enables easy gene manipulation, and the tetracycline resistance gene (tc^r). The utility of screening marker, *lacZα* with MCS, was confirmed by the blue-white screening test. Several examples of applications of gene expression in *Z. mobilis* ZM4 have been demonstrated in this article by using several new pHW20a-derived plasmids and expressing the homologous genes (*gfo* and *ppc*) and the heterologous genes (*bgl*A, *mdh*, and *fdh*1). The results show that pHW20a is a very useful new vector for construction of new *Z. mobilis* recombinant strains that will enable simultaneous co-production of biofuel and high value added products.

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KEYWORDS: *Zymomonas mobilis*; plasmid pHW20a; new vector design; expression of *bgl*A; *fdh*1; *gfo*; *mdh*; *ppc*

Introduction

Zymomonas mobilis is a facultative anaerobe and produces ethanol at high yield from such simple sugars as glucose, fructose, and sucrose. This strain, especially *Z. mobilis* ZM4, has significant metabolic advantages in terms of higher ethanol yield and specific ethanol productivity over other ethanol producing organisms currently in use such as yeast (Swings and De Ley, 1977). Construction of recombinant *Z. mobilis* strains capable of efficiently utilizing lignocellulose-derived sugar feedstocks and simultaneously co-producing ethanol and additional high value added biorefinery products has also recently become critically important to successful development of globally competitive biofuel technology (Liu et al., 2010; Rogers et al., 2007).

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Thus, it is highly desirable to design and construct a new shuttle vector having high transformation efficiency, a good screening marker, a multiple cloning sites (MCS) region, and a good stability. Attempts have been made to construct vectors with some of these desirable characteristics and generate *Z. mobilis* transformants. The pZB186 (equivalent to pZA22) was used to construct pZB5 and pZB206 for assimilation of xylose and arabinose by *Z. mobilis* recombinants (Deanda et al., 1996; Misawa and Nakamura, 1989; Zhang et al., 1995). Electroporation and $CaCl_2$ methods were used for transforming these plasmids into *Z. mobilis*. However, a high fake positive ratio of transformants or low transformation efficiency have been reported and these methods have been found to be impractical (Baumler et al., 2006).

Transconjugation method is used in this study for *Z. mobilis* transformation. It is an easy and reliable method and gives relatively high transformation efficiency. Others have also attempted to use this method for *Z. mobilis* (Cho et al., 1989; Conway et al., 1987a; Reynen et al., 1990). Conway et al. (1987a, b) have constructed a series of mobilizable plasmids with reasonably high transconjugation efficiency, typically using pLOI193. However, relatively large size of and the limited cloning sites in pLOI193 have caused some difficulties in gene manipulation. The mobilizable vector with MCS and *lacZ*α, such as pBBR1MCS-2 for over-expression of xylulokinase, was used by electroporation (Kovach et al., 1995). However, Conway et al. (1987a) have confirmed that plasmids with one *mob* gene could not be efficiently transconjugated into *Z. mobilis*.

The strategies used in this study for designing a new plasmid, pHW20a, are to include: the complete *cis*-acting elements (including *ori*V) for replication in both *E. coli* and *Z. mobilis*, two *mob* genes, a MCS region, the screening marker gene (*lacZ*α), tc^r gene, and deleting non-essential genes. The newly constructed pHW20a was evaluated for (1) transformation efficiency into *Z. mobilis* ZM4, (2) the stability of plasmid, (3) over-expression of homologous *gfo* gene encoding glucose–fructose oxidoreductase (GFOR) to increase GFOR co-production, and (4) expression of a heterologous *mdh* gene (malate dehydrogenase, MDH) to complement the missing pathway in the TCA cycle in *Z. mobilis* (Seo et al., 2005).

Materials and Methods

Microbial Strains, Plasmids, and Cell Cultures

The sources of microbial strains, their genotypes and salient characteristics, and plasmids used in this study for construction of new plasmids are given in Table I.

Isopropyl-β-D-thiogalacto-pyranoside (IPTG), antibiotics (Km, Amp, Cm, Tc), oxaloacetic acid (OAA), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), *N*-morpholinoethanesulfonic acid (MES), nalidixic acid (NA), and *p*-nitrophenol were purchased from Amresco Chemical, Inc. (Cleveland, OH) or Acros Organics (Geel, Belgium). Bovine serum albumin (BSA) and NADH from Sigma–Aldrich (St. Louis, MO), and all other analytical grade chemicals from Sinopharm Chemical Reagent (Shanghai, China). Appropriate antibiotics were added to the medium at 50 $\mu g\,mL^{-1}$ for each Cm, Km, Amp, and 20 $\mu g\,mL^{-1}$ for Tc, depending on the antibiotic resistance of the recombinant strains. The concentrations of other chemicals used were: 50 $\mu g\,mL^{-1}$ X-Gal; 40 $\mu g\,mL^{-1}$ for NA, and 25 $\mu g\,mL^{-1}$ for IPTG.

The Luria broth (LB) was used for *E. coli* cultivation in a rotating incubator at 37°C and 220 rpm. The seed preparation for *Z. mobilis* and the recombinant strains derived from it were incubated in RM medium containing: 20 $g\,L^{-1}$ of glucose, 2 $g\,L^{-1}$ of KH_2PO_4, and 10 $g\,L^{-1}$ of yeast extract. The pH value was adjusted to 6.0 before sterilization. The fermentation medium contained: glucose, 100 $g\,L^{-1}$; yeast extract, 5 $g\,L^{-1}$; KH_2PO_4, 1 $g\,L^{-1}$; $(NH_4)_2SO_4$, 1 $g\,L^{-1}$; $MgSO_4{\cdot}7H_2O$, 0.5 $g\,L^{-1}$; and pH value was adjusted to 6.0. The LB agar plate medium containing IPTG, X-Gal, and Tc is abbreviated as LIXT.

The *Z. mobilis* cell cultures were incubated at 30°C anaerobically. The inoculum seed in vials was prepared using 1.0 mL aliquots of seed culture containing 30% (w/v) glycerol and stored at −80°C freezer. For the first-stage start-up inoculum culture, 20 mL RM medium was inoculated with one vial of the seed until the optical density (OD) of the cells reached about 2.0 spectrophotometer reading at 600 nm. For the second-stage inoculum culture, 100 mL fresh RM medium was inoculated with 1% (v/v) inoculum from the start-up inoculum culture and incubated until the OD_{600nm} reading reached 1.0–1.1 range. One liter fermentation medium in 3-L fermentor (3BG, Baoxing Biotech, Shanghai, China), was inoculated with 10% (v/v) second-stage inoculum culture. Fermentation media were sterilized at 115°C for 20 min and 10 $\mu g\,mL^{-1}$ Tc added before inoculation for recombinant strains. The anaerobic fermentation was carried out at 200 rpm agitation speed using nitrogen gas flush-out method (>99.9% purity). The pH value of the medium was automatically controlled at 6.0 with 5 M NaOH.

DNA Techniques

Enzymes and other reagents were purchased from Fermentas International, Inc. (Burlington, ON, Canada). Plasmid extraction and gel purification were carried out using Omega Plasmid Mini Kit and Omega Gel Extraction Kit (Omega Biotek, Inc., Norcross, GA). The genomic DNA of *Z. mobilis* ZM4 and *E. coli* BL21 (DE3) were prepared using Qiagen DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA). The genetic manipulations and SDS–PAGE analysis were routinely performed according to the well-established protocols (Sambrook et al., 1989) or the manufacturers' suggested instructions provided. The cell lysates were analyzed by 12% (w/v) SDS–PAGE, and the SDS–PAGE gels was pictured and analyzed by ImageQuant™ LAS 4010 (GE Healthcare Life Sciences, Piscataway, NJ). The oligonucleotides used for DNA amplification are listed in Table II.

Table I. Microbial strains and plasmids 1 used in this study.

Strains and plasmids	Genotype and/or salient characteristics[a]	Source/reference
Strains		
Z. mobilis ZM4	Wild-type strain, ATCC 31821	Purchased from ATCC
Z. mobilis ZM6	Wild-type strain, ATCC 29191	Purchased from ATCC
E. coli DH5α	F$^-$, φ80Δ*lacZ*ΔM15 Δ (*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17 (rk^-, mk^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Grant et al. (1990)
E. coli BL21 (DE3)	F$^-$ *ompT hsdSB* (rB^- mB^-) *gal dcm* (DE3)	Novagen
E. coli S 17-1 λ π	*Pro*, *res*$^-$, *mod*$^+$; chromosomal integrated RP4, 2-*Tc*::Mu-*Km*::Tn7;*Tp*, *sm*	Simon et al. (1983)
Bacillus polymyxa	Wild-type strain, CGMCC 1.794	Purchased from CGMCC
Plasmids		
pUC19	*Ori* pMB1, *amp*^{r}, MCS, *lacZ*α; cloning vector	Yanisch-Perron et al. (1985)
pET-28a (+)	*Km*^{r}, *ori* pMB1, T7 promoter/terminator	Novagen
pBR322	*Amp*^{r}, *tc*^{r}, *ori* pMB1; *tc*^{r} donor vector	Sutcliffe (1979)
pBBR1MCS-2	*Km*^{r}, *ori*V, *mob* (RK2), 5.1 kb	Kovach et al. (1995)
pBBR1MCS-2-*tc*^{r}	*Tc*^{r}, pBBR1MCS-2-derived plasmid, 5.2 kb	In this study
pLOI193	*Cam*^{r}, *tc*^{r}, *mob* (RSF1010), *mob* (RP4), ColE1 and *ori*V	Conway et al. (1987a)
pHW20a	*Tc*^{r}, *mob* (RP4), *mob* (RSF1010), *lacZ*α, MCS and o*ri*V	In this study
pHW20a-*gfo*	Over-express gfo_{Zm} with its native promoter in *Z. mobilis*	In this study
pUC19-*mdh*	Plasmid with mdh_{Ec} subcloned into pUC19	In this study
pET-28a-*mdh*	Express mdh_{Ec} in *E. coli* BL21 (DE3) for activity confirmation	In this study
pUC19DN	pUC19-derived plasmid without NdeI site	In this study
pUC19DN-P*ppc*	Ppc_{Zm} promoter was subcloned into pUC19DN	In this study
pUC19DN-P*gap*	Gap_{Zm} promoter was subcloned into pUC19DN	In this study
pET-28a-*bgl*A	Express $bglA_{Bp}$ in *E. coli* BL21 (DE3) for activity confirmation	In this study
pET-28a-*fdh*1	Express $fdh1_{Sc}$ in *E. coli* BL21 (DE3) for activity confirmation	Provided by Dr. L.Q. Fan
pUC19DN-P*ppc-mdh*	Ligation of mdh_{Ec} with ppc_{Zm} promoter	In this study
pHW20a-*mdh*	Express mdh_{Ec} in *Z. mobilis* with ppc_{Zm} promoter of ZM4	In this study
pUC19-*tc*^{r}	Plasmid subcloned *tc*^{r} complete gene from pBR322	In this study
pUC19-*mob* (RP4)^{pre}	Plasmid subcloned *mob* (RP4) from pLOI193	In this study
pUC19-*mob* (RP4)	Fragment flanked by two *Eco*RI sites in pUC19-*mob* (RP4)^{pre} was deleted	In this study
pUC19-*mob* (RP4)-*tc*^{r}	Plasmid with combined *mob* (RP4) and *tc*^{r} genes	In this study
pHW10a	*Tc*^{r}, *mob* (RP4), *mob* (RSF1010) and o*ri*V	In this study
pHW10a-SCC	*Sm*^{r}*A*, *sm*^{r}*B*, *cm*^{r}, *tc*^{r}, *mob* (RP4), *mob* (RSF1010) and o*ri*V	In this study
pHW20a-*bgl*A	Express $bglA_{Bp}$ in *Z. mobilis* ZM4 with gfo_{Zm} promoter of ZM4	In this study
pUC19DN-P*gap-fdh*1	Ligation of $fdh1_{Sc}$ with gap_{Zm} promoter	In this study
pHW20a-*fdh*	Express $fdh1_{Sc}$ in *Z. mobilis* ZM4 with gap_{Zm} promoter of ZM4	In this study
pHW20a-*ppc*	Express ppc_{Zm} in *Z. mobilis* ZM4 with ppc_{Zm} native promoter	In this study

[a]Host organism designation in subscripts of genes used: *Zm*, *Z. mobilis* ZM4; *Ec*, *E. coli* BL21 (DE3); *Bp*. *B. polymyx* (CGMCC 1.974); *Sc*, *Saccharomyces cerevisiae*.

Construction of Vector pHW20a

The details for constructing pHW20a (9.9 kb) is shown in Figure 1 and described as follows:

Step 1: The *mob* (RP4) with an incomplete *tc*^{r} promoter (P*tc*^{r}) containing a 2.0 kb *mob* gene of RP4 (Access No.: L27758.1), which ensured mobilizable RP4 (Simon et al., 1983), was subcloned into pUC19 via homologous *Hin*dIII cohesive ends to generate pUC19-*mob* (RP4)^{pre}.

Step 2: Plasmid pUC19-*mob* (RP4)^{pre} was digested by *Eco*RI to eliminate one *Hin*dIII site. The linearized plasmid was cyclized by T4 DNA ligase to generate the pUC19-*mob* (RP4).

Step 3: The complete *tc*^{r} gene was obtained by PCR from pBR322 and was subcloned into pUC19 at the *Eco*RI and *Pst*I sites to generate pUC19-*tc*^{r}.

Step 4: The *tc*^{r} gene without P*tc*^{r} was obtained from pUC19-*tc*^{r} by digestion with *Hin*dIII and *Eco*RI. The truncated incomplete *tc*^{r} was inserted into pUC19-*mob* (RP4) at the truncated *Hin*dIII site after pUC19-*mob* (RP4) was sequentially treated with *Hin*dIII and T4 DNA polymerase. The achievement of the Tc resistance in the newly constructed pUC19-*mob* (RP4)-*tc*^{r} indicated the correct ligation of two DNA fragments with a complementation of the promoter.

Step 5: The 5.7 kb fragment containing *mob* (RSF1010), *ori*V replicon, and genes necessary for the replication in gram-negative bacteria was obtained by digesting pLOI193 with *Hin*cII and *Pst*I. The fragment containing *mob* (RP4) and *tc*^{r} genes was generated from pUC19-*mob* (RP4)-*tc*^{r} by *Nco*I and *Bst*BI. The two DNA fragments were both treated by T4 DNA polymerase, and then they were ligated to generate pHW10a. The newly constructed plasmids were digested with *Sca*I and *Bam*HI for confirmation of the ligation direction as shown in Figure 1.

Step 6: The *lacZ*α with four extra cloning sites designed in the primers was obtained from pUC19 by PCR and directly inserted into pHW10a (9.5 kb) at *Sac*I site. The plasmids

Table II. Oligonucleotides 1 used in this study.

No.	Primers[a]	Sequence (5′ → 3′)
1	*Mdh*-S	CGGGATCCCATATGAAAGTCGCAGTCCTCGG
2	*Mdh*-A	GGAATTCTTACTTATTAACGAACTCTTCGCCC
3	*Gfo*-S	CGGAATTCTCGAAATTAACGATCACCCAC
4	*Gfo*-A	GCTCTAGACCATGGTCAATAACCACCCTGACGG
5	P*ppc*-S	CGCGGATCCGCGGTGGCGTCTATGAGAGCATG
6	P*ppc*-A	CGGAATTCCCGGGCATATGAAACCATCCCTTTGCCTA
7	*Tc*^{r}-S	AACTGCAGCATGTTTGACAGCTTATCATCG
8	*Tc*^{r}-A	GGAATTCCATGGTTCCATTCAGGTCGAGGTG
9	*LacZ*-S	AGATCTACGCGTCTATGCGGCATCAGAGCAG
10	*LacZ*-A	GCGGCCGCCTCGAGTAGCTCACTCATTAGGCACCC
11	QRT-*gap*-S	GATATCGTGATGGAATGCAC
12	QRT-*gap*-A	GAGCCAAGCAGTTGGTGG
13	QRT-*ppc*-S	GCCCTGAATCCATTACGA
14	QRT-*ppc*-A	GCTTGAACCATTCCTCCA
15	PS*gfo*-S	CGGAATTCTCGAAATTAACGATCACCCAC
16	PS*gfo*-A	CCTGCGGAAATTGAAAAATAGTAAGCGTCGCTGCCTGAAG
17	*Bgl*A-S	CTTCAGGCAGCGACGCTTACTATTTTTCAATTTCCGCAGG
18	*Bgl*A-A	GCTCTAGATTAGCGTCTAGTCTCCAACCAG
19	P*ppc-ppc*-S	GCTCTAGACTCGAGTGGCGTCTATGAGAGCATG
20	P*ppc-ppc*-A	GGGGTACCGGATCCTCAACCGCTGTTGCGAAGTG
21	P*gap*-S	CGCGGATCCGCGGTTGGACTTTGTTCGATCAAC
22	P*gap*-A	CGGAATTCCCGGGCATATGTTTATTCTCCTAACTTATTAAGTAGC
23	*Fdh*1-S	GCTCTAGACATATGTCGAAGGGAAAGGTTTTG
24	*Fdh*1-A	CGAATTCAGATCTGCGGCCGCTCTTATTTCTTCTGTCCATAAGCTC

QRT, oligonucleotides used for qRT-PCR; S, sense primer strand; and A, anti-sense primer strand.
[a]Words in italics, target and reference genes.

identified as the structure shown in Figure 2 were selected from blue colonies appeared on LIXT agar plates.

The plasmids constructed in every step were identified by enzyme digestion and PCR. DNA sequencing was used for the identification of the cloning results of *mob* (RP4), *tc*^{r}, and *lacZ*α.

Construction of New Plasmids for Studies on Transconjugation Efficiency and Stability

In order to study the effects of *mob* gene copy number, plasmids of reduced size obtained by deleting non-essential genes, and heterologous genes on the transconjugation efficiency and stability of pHW20a in *Z. mobilis*, several new plasmids were constructed, and homologous and heterologous genes of interest were selected, cloned, and expressed in *Z. mobilis*. The detailed information for construction of these plasmids is given in the Supplemental Information Section.

Transconjugation Method

In order to evaluate the key factors affecting the transconjugation efficiency, the transformation efficiencies of pLOI193 and the new plasmids constructed shown in Figure 2 were evaluated and compared. Transformation of plasmids into *Z. mobilis* was performed using *E. coli* S 17-1 λ π and the biparental transconjugation method and the transformation efficiencies of these plasmids were estimated by using the method proposed by Conway et al. (1987a).

Sample Preparation and Enzyme Assay

The whole broth samples (20 mL) were taken from the fermentor, centrifuged at 10,000*g* for 5 min at 4°C, the collected cells were washed twice with 20-mL of pre-chilled buffer corresponding to the enzyme assay reaction system, and the collected cell samples were stored at −80°C for enzyme activity analysis later. The cells were resuspended with the cold buffer and disrupted by sonication at 200 W for 5 min (20 cycles, 3 s on and 12 s off) in ice-water bath. The disrupted cells were centrifuged at 15,000*g* for 30 min at 4°C and the supernatant was collected. Protein concentration was determined by Bradford protein assay using BSA calibration curve.

Activities of MDH in *Z. mobilis* ZM4 and *E. coli* BL21 (DE3) were assayed with 1 mM sodium oxaloacetate and 0.4 mM NADH as substrates at 30°C (Park et al., 1995). The GFOR activity was assayed at 25°C according to the method described earlier (Zachariou and Scopes 1986). The β-glucosidase activity was assayed in 50 mM citrate buffer (pH 6.0) with PNPG as the substrate at 37°C according to a previous report (Gonzalez-Candelas et al., 1989). The NAD^+-dependant formate dehydrogenase activity was estimated by the reduction rate of NAD^+ assayed at 340 nm by the method reported (Berríos-Rivera et al., 2002). The

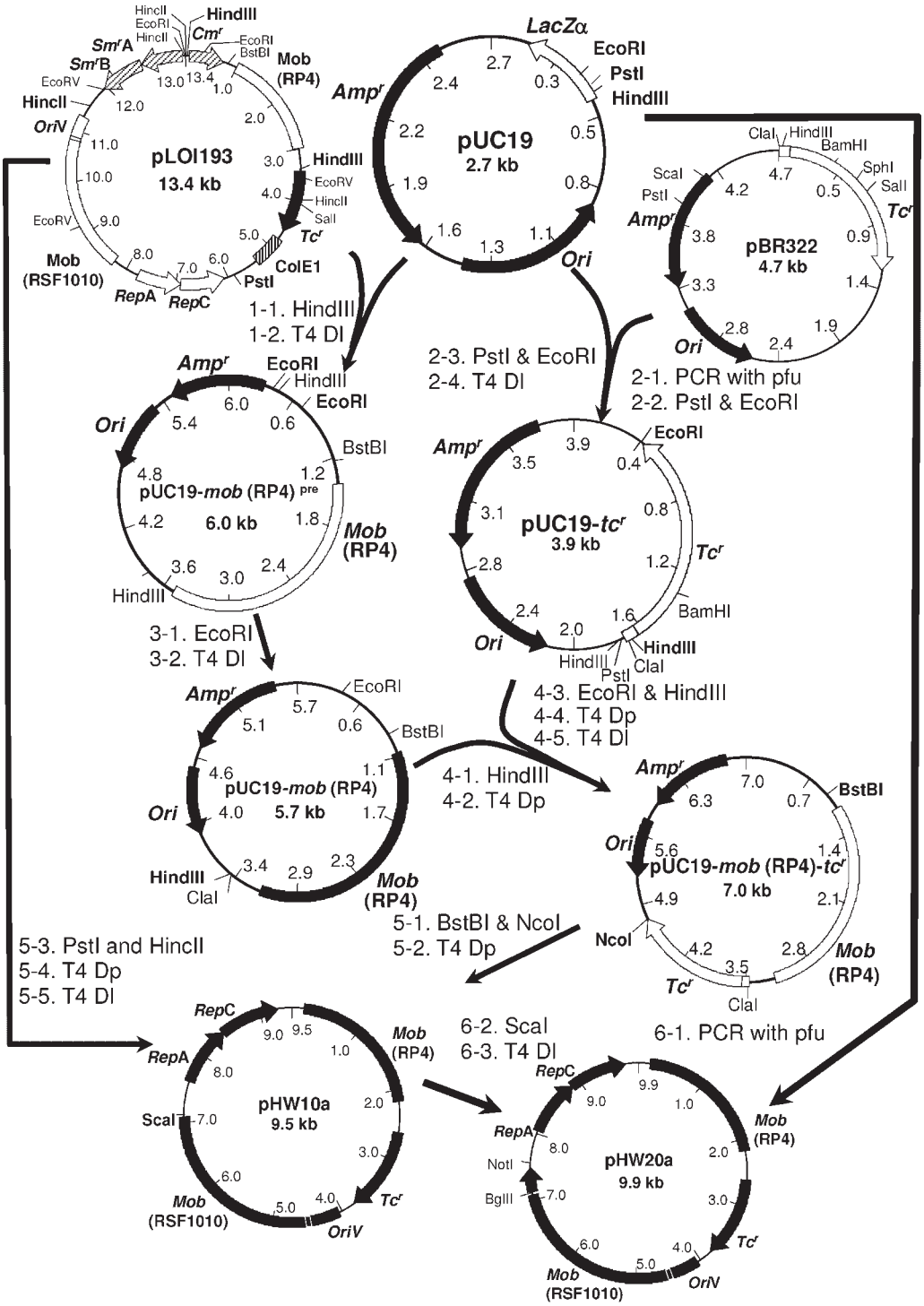

Figure 1. Design and construction of pHW20a. The plasmids designed and constructed, including the names, sizes, *cis*-acting elements, and the procedure are shown. The designations of procedural steps and enzymes used: the first number for gene manipulation steps and the hyphenated second number after each step for the restriction enzymes used in sequence. For example, *Hin*dIII (1–1) and T4 D1 (1–2) are sequentially used for the first step. White blank arrows and bands indicate the DNA fragments to be sliced for the immediately following gene manipulation step in construction of the plasmids. The striped bands in pLOI193 plasmid map shows non-essential genes to *Z. mobilis* for the transconjugation, replication, and screening. The overlap region of *oriV* with *mob* (RSF1010) is shown as a white blank bands. Restriction sites are shown in boldface indicating that the sites will be used in the next or immediately following gene manipulation step. More detailed description of procedure should be referred to the Materials and Methods Section.

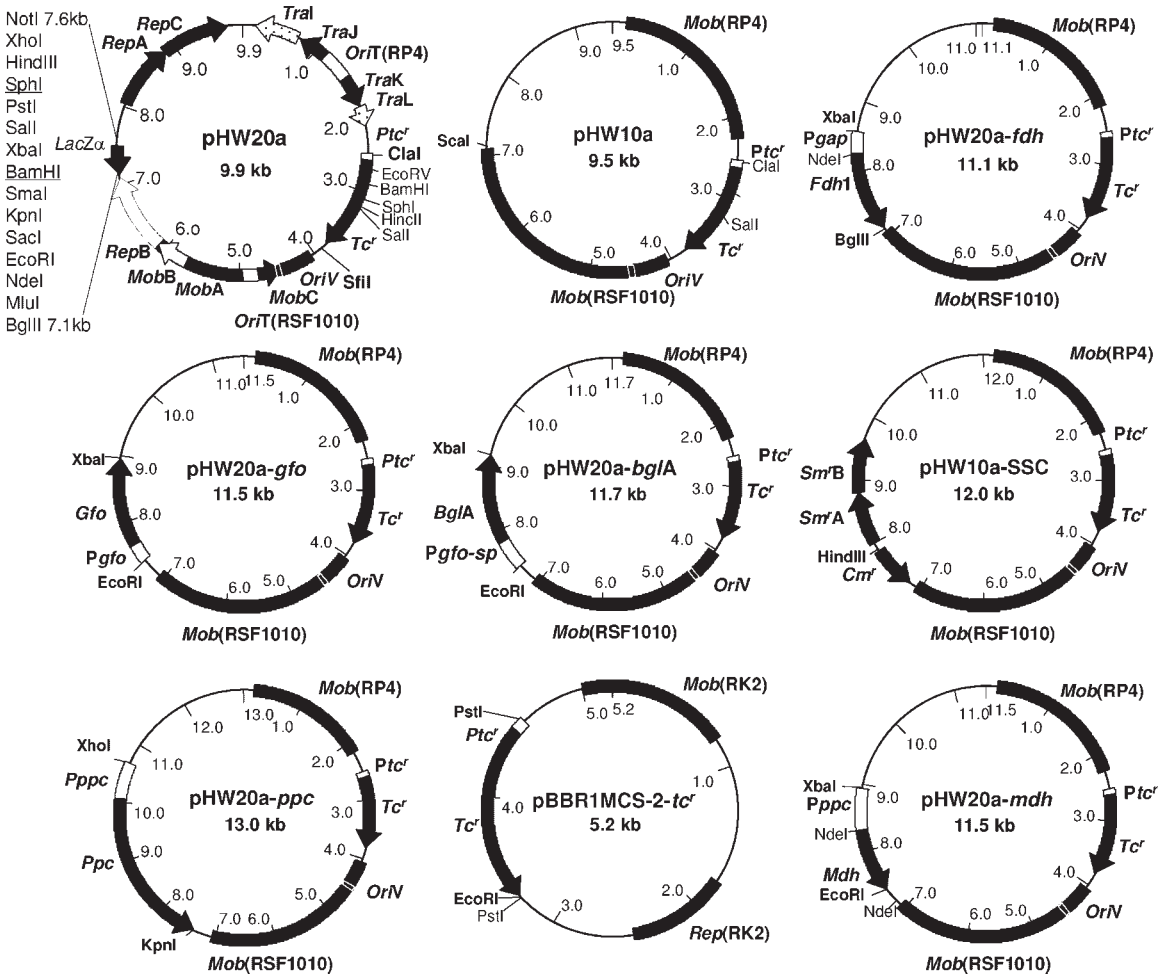

Figure 2. Detailed maps of pHW20a and other newly constructed plasmids used for analyses of transconjugation efficiency and stability. Detailed description of pHW20a: The *rep*A, *rep*B, and *rep*C genes encode proteins participating in the replication of pHW20a. The *Mob*A, *mob*B, and *mob*C encode proteins necessary for the mobilization of pHW20a and they constitute the *mob* gene of RSF1010 with *Ori*T (RSF1010). White blank arrow, *OriV*, designate the plasmid replication region. The white blank arrows with dots indicate the incomplete gene segments. The *Tra*I, *tra*J, *tra*K, *tra*L, and *ori*T (RP4) constitute the *mob* gene of RP4. The shaded boxes indicate two gene segments, *ori*T, from RSF1010 and RP4 for transfer replication. The underlined restriction endonucleases sites indicate those sites are not unique in pHW20a, and their counterpart in *tc*^{r} are also marked in the plasmid map. Other plasmids: The arrows indicate the direction of the genes (5′ → 3′); the black bands indicate the *mob* genes and *oriV* cassettes. The promoter regions in the plasmids are shown as white blank boxes. The overlap region of *mob* (RSF1010) with *oriV* is shown as a black band. The *mob*B and *rep*B overlapped with *mob*A are shown in white blank arrows. See also the Results and Discussion Section for more details.

standard enzyme activity units are used for MDH, GFOR, β-glucosidase, and FDH.

Analyses of Glucose, Ethanol, and Cell Concentration

Cell concentration was monitored by measuring OD at 600 nm and the dry cell mass (DCM) was estimated according to the method reported earlier (Sáez-Miranda et al., 2006). Glucose and ethanol were analyzed using high-performance liquid chromatography (LC-20AD, refractive index detector RID-10A, Shimadzu, Japan) with a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) at the column temperature 65°C. The mobile phase was 5 mM H_2SO_4 at the flow rate of 0.6 mL min^{-1}. All cell samples were centrifuged at 15,000*g* for 5 min at 4°C, and the supernatant was filtered through a 0.22 μm filter for glucose and ethanol analysis.

Measurement of *gap* and *ppc* Transcription by qRT-PCR

In order to confirm that the P*ppc* is a relatively weak promoter, as was observed during the expression of *mdh* in *Z. mobilis* (pHW20a-*mdh*), the transcription levels of target *ppc* gene were estimated and normalized to those of endogenous reference *gap* gene (encoding the glycerol phosphate dehydrogenase in ED pathway, GAPDH) at the same thermal cycling conditions. The cell extract samples were taken during fermentation and used for quantitative

real-time PCR (qRT-PCR) analysis. The relative gene transcription data were analyzed using qRT-PCR and the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (2001).

The total RNA from *Z. mobilis* ZM4 wild strains was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and the residual DNA removed using RNase-free DNase I (Fermentas Life Science, Burlington, Ontario, Canada). The reverse transcription was performed using total RNA and the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas Life Science). The cDNA was quantified using a Mastercycler® ep realplex 2S detection system (Eppendorf AG, Barkhausenweg, Hamburg, Germany). The PCR reactions were carried out using and MaximaTM SYBR Green qPCR Master Mix (MBI Fermentas) with the *gap* primer pair and the *ppc* primer pair, respectively.

Stabilities of Plasmids in *Z. mobilis* ZM4

The stabilities of pHW20a and its derivatives for gene expressions were all evaluated by estimating the ratio of the plasmid harboring cells to the total cell population during the prolonged cultures. The samples of cells grown in RM medium without Tc were taken for the stability analysis. Serial batch cultures were used by inoculating the next batch with 1% inoculum (v/v) taken from the previous batch when its cell concentration reached $OD_{600} = 1.0$. The ratio of the number of colonies appeared on RM agar plates containing Tc to that on the RM agar plates without Tc was estimated as the fraction of plasmid harboring cells.

Results and Discussion

Design and Characterization of New Plasmid pHW20a

The map of newly constructed plasmid pHW20a is shown in Figures 1 and 2 and its complete sequence analyzed is given in the Supplemental Figure 1. The size of pHW20a is reduced to 9.9 kb by deleting the selected non-essential genes (smrA, smrB, cmr, and ColE1) from pLOI193 (13.4 kb). It contains one replication region (*ori*V), tetracycline resistance gene (tc^r), two *mob* genes (*mobA* from RP4 and *mobB* from RSF1010), one blue-white screening marker (*lac*Zα), and the MCS region within *lac*Zα. The cloning of *lac*Zα containing MCS in pHW20a was confirmed by sequence analysis, α-complementation test, and insertion mutation test by cloning *gfo* and *mdh* genes in *E. coli* DH5α with LIXT agar plates (data not shown). The use of *lac*Zα made gene manipulation much easier in *E. coli* system, such as *E. coli* DH5α.

The MCS in pHW20a were confirmed by restriction endonucleases digestion in Figure 3 and the *lac*Zα sequence data (data not shown). There were 12 unique restriction sites available for cloning by blue-white screening using *lac*Zα. Because *lac*Zα promoter is recognizable by *Z. mobilis* ZM4

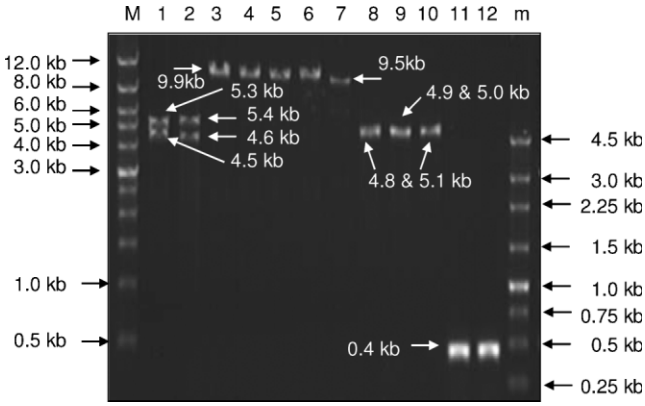

Figure 3. Agarose gel analysis of restriction endonuclease digested plasmid pHW20a. Lane M, wide range marker (500 bp to 12 kb, Takara Bio Inc., Otsu, Shiga, Japan); lane 1, pHW20a (*Cla*I/*Nde*I, 5.3 and 4.6 kb); lane 2, pHW20a (*Bam*HI, 5.4 and 4.5 kb); lane 3, pHW20a (*Cla*I, 9.9 kb); lane 4, pHW20a (*Hin*dIII, 9.9 kb); lane 5, pHW20a (*Eco*RI, 9.9 kb); lane 6, pHW20a (*Xba*I, 9.9 kb); lane 7, pHW10a (*Sca*I, 9.5 kb); lane 8, pHW20a (*Cla*I/*Hin*dIII, 4.8 and 5.1 kb); lane 9, pHW20a (*Cla*I/*Xba*I, 4.9 and 5.0 kb); lane 10, pHW20a (*Cla*I/*Eco*RI, 4.8 and 5.1 kb); lane 11, *lac*Zα PCR products with pHW20a as a template (0.4 kb); lane 12, *lac*Zα PCR products with pUC19 as a template (0.4 kb); lane m, Marker (DL 250 bp, Takara Biotechnology Co. Ltd).

(Carey et al., 1983; Cornelis et al., 1978), *Not*I and *Xho*I located at the upstream of *lac*Zα in pHW20a could also be used to remove and/or replace the *lac*Zα promoter (P*lac*Zα) with another. This option will be equally useful, if any undesired effect of P*lac*Zα promoter on the transcription of cloned genes in pHW20a is found. The *Sfi*I and *Cla*I restriction sites flanking the tc^r gene could also be used to replace tc^r readily with other resistance genes in pHW20a for a wide range of application of gram-negative host organisms as an alternate host organism.

Expression of Native *gfo* Gene in *Z. mobilis* (pHW20a-*gfo*)

GFOR is an important enzyme participating in the osmotic regulation of *Z. mobilis* by producing sorbitol (Loos et al., 1994) and it has a potential practical application for simultaneous co-production of sorbitol, ethanol, and gluconic acid (Jonas and Silveira, 2004). Because of complex mechanisms involved in the *gfo* post-translational process, the over-expression of *gfo* have not been fully realized in *E. coli* (Thomas et al., 1997). In order to substantially increase the GFOR production, homologous *gfo* gene was amplified in *Z. mobilis* ZM4 (pHW20a-*gfo*), as an example of newly designed vector, pHW20a, for over-expression of *gfo* gene. Figure 2 shows the map of pHW20a-*gfo*, which has been confirmed by restriction enzyme digestion and PCR results (data not shown).

The over-expression of *gfo* in *Z. mobilis* ZM4 (pHW20a-*gfo*) was compared with ZM4 (pHW20a) as the control and the results are shown in Figure 6B and A, respectively. For the *Z. mobilis* ZM4 (pHW20a-*gfo*), the GFOR enzyme yield and specific activity obtained (837 ± 66 U g^{-1} DCW and

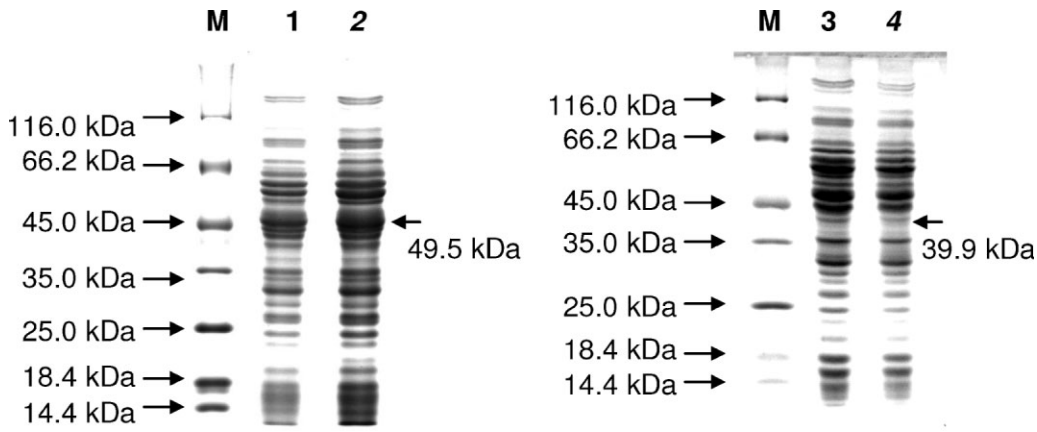

Figure 4. SDS–PAGE photographs of *Z. mobilis* ZM4 (pHW20a-*gfo*) and *Z. mobilis* ZM4 (pHW20a-*mdh*). Lane M: unstained protein molecular weight marker (14.4–116.0 kDa, Fermentas Life Science, Burlington, Ontario, Canada); lane 1: *Z. mobilis* ZM4 (pHW20a) (15 μg protein); lane 2: *Z. mobilis* ZM4 (pHW20a-*gfo*) (15 μg protein); lane 3: *Z. mobilis* ZM4 (pHW20a) (20 μg protein); lane 4: *Z. mobilis* ZM4 (pHW20a-*mdh*) (20 μg protein). The construction method for these plasmids is given in the Supplemental Section.

$6.80 \pm 0.15\ U\ mg^{-1}$ at the 16-h) is about twofold greater than that of the ZM4 (pHW20a) ($394 \pm 40\ U\ g^{-1}$ DCW and $3.20 \pm 0.07\ U\ mg^{-1}$ at the 13-h) and this result was also confirmed by the SDS–PAGE analysis as shown in Figure 4. The cell yields of both recombinant strains, *Z. mobilis* ZM4 (pHW20a-*gfo*) and (pHW20a), are about same, 3.29 and $3.52\ g\ L^{-1}$, respectively. The ethanol yields based on glucose substrate for both recombinant strains are also found to be practically the same, about 93% of the theoretical ethanol yield. These results indicate that the metabolic pathways linked to ethanol biosynthesis have not been adversely affected by the over-expression of GFOR. In the wild strain, *Z. mobilis* ZM4, GFOR expression by homologous *gfo* gene was about 1% of the total soluble protein in cell lysate (Kanagasundaram and Scopes, 1992). When the *gfo* gene was over-expressed in the *gfo* amplified *Z. mobilis* ZM4 (pHW20a-*gfo*) recombinant, the cell growth, ethanol production, and the glucose assimilation all showed a significant delay (Fig. 6B) while the GFOR-specific activity increased about twofold as compared to the metabolic pattern shown by the ZM4 (pHW20a) control strain (Fig. 6A). This observation of distinctly different metabolic patterns between these two strains suggests that the GFOR over-expression has caused a significant metabolic stress on the host recombinant strain, *Z. mobilis* ZM4 (pHW20a-*gfo*) (Bentley et al., 1990; Glick, 1995; Ow et al., 2006). When *Z. mobilis* ZM4 (pHW20a-*gfo*) recombinant cells were used for sorbitol production, the sorbitol productivity increased by about twofold compared to that of the wild strain at the same cell-loading in the bioreactor and under the same operating conditions (Liu et al., 2010). Similarly, the GFOR-specific activity ($5.05 \pm 0.10\ U\ mg^{-1}$) obtained from *Z. mobilis* ZM6 (pHW20a-*gfo*) was 1.6-fold higher than that ($3.16 \pm 0.14\ U\ mg^{-1}$) obtained from the reference recombinant strain ZM4 (pHW20a), but slightly lower than that ($6.80 \pm 0.15\ U\ mg^{-1}$) obtained from ZM4 (pHW20a-*gfo*) recombinant strain with the amplified *gfo* gene. These results have shown that the homologous *gfo* gene was successfully over-expressed by the amplified *gfo* gene in both recombinants, *Z. mobilis* ZM4 (pHW20a-*gfo*) and ZM6 (pHW20a-*gfo*).

Expression of *mdh* Gene in *Z. mobilis* ZM4 (pHW20a-*mdh*)

The *mdh* gene encodes MDH which carries out a reversible reaction for the formation of either malate or OAA in TCA

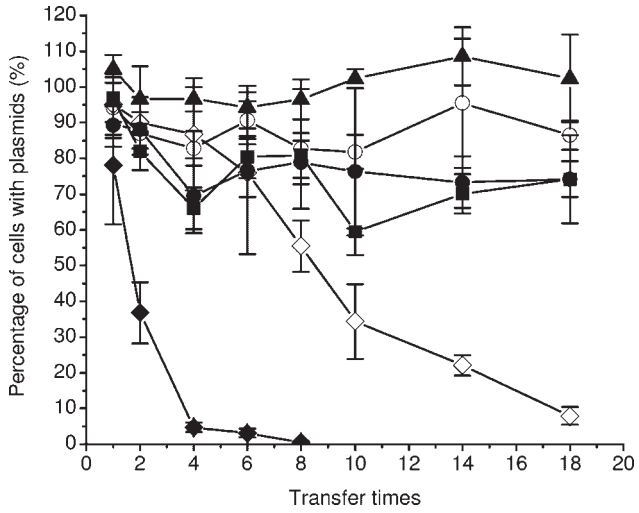

Figure 5. Stabilities of new plasmids constructed in *Z. mobilis* ZM4. The plasmid harboring cells as a fraction of total cell population (including both the plasmid harboring and plasmid free cells) in the culture was determined as a function of fermentation time when grown on RM agar plates with and without tetracycline (Tc). The symbols: (○) for *Z. mobilis* ZM4 (pHW20a); (▲) for *Z. mobilis* ZM4 (pHW20a-*fdh*); (■) for *Z. mobilis* ZM4 (pHW20a-*bgl*A); (●) for *Z. mobilis* ZM4 (pHW20a-*mdh*); (◇) for *Z. mobilis* ZM4 (pHW20a-*ppc*); and (◆) for *Z. mobilis* ZM4 (pHW20a-*gfo*). The average values from the triplicate experimental data are given and the error bars indicate standard deviation.

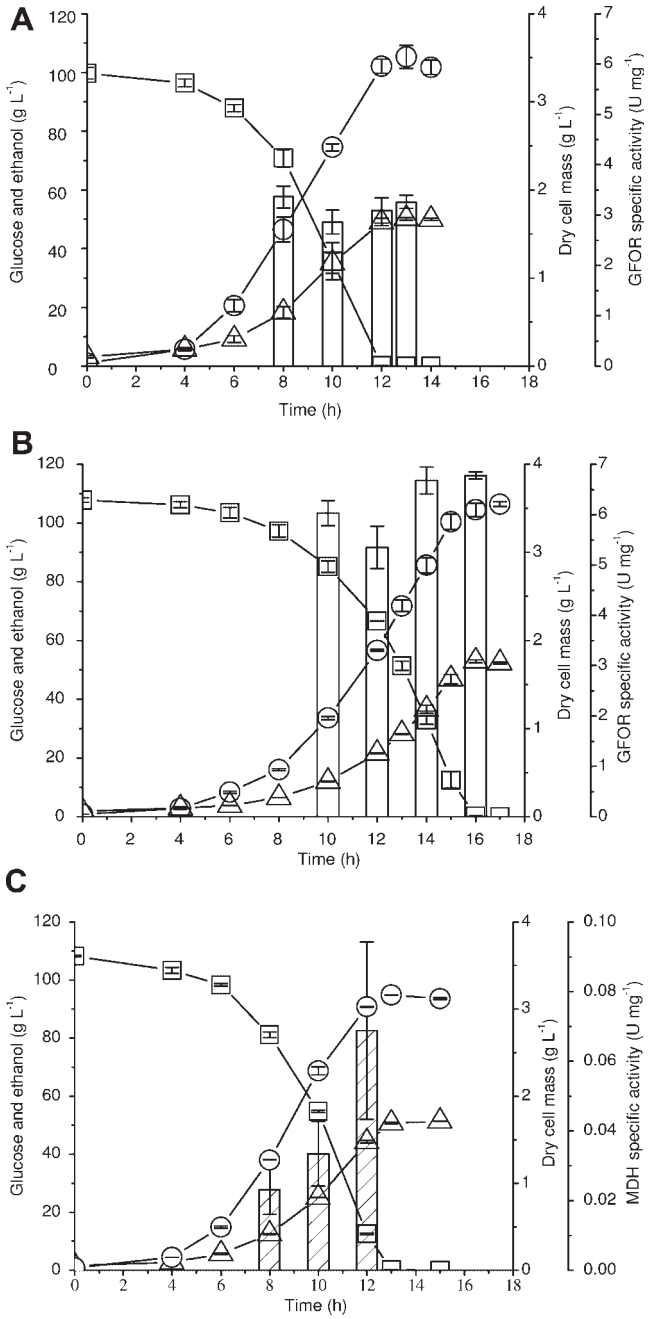

Figure 6. Batch fermentation profiles for (**A**) *Z. mobilis* ZM4 (pHW20a), (**B**) *Z. mobilis* ZM4 (pHW20a-*gfo*), and (**C**) *Z. mobilis* ZM4 (pHW20a-*mdh*). Symbols: (○), dry cell mass; (□), glucose concentration; (Δ), ethanol concentration; open bar columns, specific activity of GFOR; and striped bar columns, specific activity of MDH. The average values from triplicate experimental data are given and the error bars indicate standard deviation.

cycle. However, this pathway is absent in *Z. mobilis* ZM4 (Seo et al., 2005). The malate is produced from pyruvate by malic enzyme (ME) in *Z. mobilis*. An expression of heterologous *mdh* in *Z. mobilis* ZM4 recombinant was also evaluated. In order to minimize potential adverse effect of the heterologous *mdh* on cellular physiology of host cells, a putative weak promoter, P*ppc*, was used for the regulation of *mdh* expression. The PEPCase, encoded by *ppc*, plays an important role in carbon fixation in C4 and Crassulacean Acid Metabolism plants (O'Leary, 1982). Because of very low carbon flux ratio of PEPCase pathways to that of the ED pathway for ethanol production in *Z. mobilis* ZM4, the P*ppc* is recognized as a weak promoter. The map of pHW20a-*mdh* is also shown in Figure 2.

The results of the *mdh* expression in terms of MDH-specific activity in the *Z. mobilis* ZM4 (pHW20a-*mdh*) are shown in Figure 6C. No MDH activity was detected from *Z. mobilis* ZM4 (pHW20a), confirming that there was no endogenous *mdh* gene in *Z. mobilis* ZM4. The similar results were also reported earlier for the wild strains of *Z. mobilis* (Bringer-Meyer and Sahm, 1989; Seo et al., 2005). The maximum MDH-specific activity measured was $0.07 \pm 0.002\ \mathrm{U\ mg^{-1}}$ at 12-h when the glucose was exhausted and it accounted for only about 0.01% of the soluble protein in the cell lysate. The specific activity of purified MDH from *E. coli* was reported as $950.4\ \mathrm{U\ mg^{-1}}$ (Murphey et al., 1967). The results confirmed an expression of *mdh* from the *Z. mobilis* ZM4 (pHW20a-*mdh*) and the relatively low MDH activity observed confirmed a successful design with the weak promoter, P*ppc*. The MDH band at 39.9 kDa in the SDS–PAGE of *Z. mobilis* ZM4 (pHW20a-*mdh*) is very faint and indistinguishable from the control strain, indicating a very weak expression of heterologous *mdh* in the recombinant strain (Fig. 4).

Transcription and Expression of *ppc* Relative to *gap*

Because the estimation of PEPCase activity in *Z. mobilis* cell lysate may not be accurate due to the interference from pyruvate carboxylase and PEP carboxykinase (Bringer-Meyer and Sahm, 1989), the transcription level of *ppc* was analyzed using *gap*, a gene located in the glycolysis pathway in *Z. mobilis* wild strain, as the endogenous reference gene (Conway et al., 1987c; Pawluk et al., 1986) to confirm that the P*ppc* promoter is relatively weak in *Z. mobilis* ZM4 (Fig. 7). The relative transcription level of *ppc* was analyzed using qRT-PCR.

The maximum relative transcription level of *ppc* observed was in the middle of the exponential phase (at the 8-h), that is about 3.16 times higher than the initial transcription level of the seed culture (at 0-h) and about 1.6 times higher than the level at the 4-h. As the culture approached the stationary phase, the relative transcription level of *ppc* decreased to about 1.65 that is approximately the same as that detected at the 4-h. The numbers given in the bar columns in Figure 7 represent the ratios of *ppc/gap* transcriptions that is equivalent to *ppc* normalized with respect to *gap*. Compared to the transcription level of the internal control reference gene, *gap*, the transcription levels of *ppc* are low in the range of only 1.8–5.7% relative to the *gap* transcription. The maximum transcription level of *ppc* was only 5.7% that of *gap* at about 8-h. The mRNA of *ppc* remained at a very low level and this could partially explain the high ethanol yield

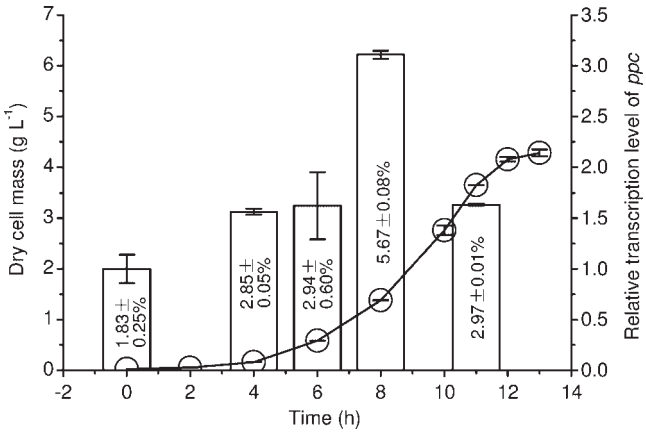

Figure 7. The qRT-PCR analysis of relative ratios of transcription and expression levels of *ppc*/*gap*. The *gap* was used as the endogenous reference gene. Symbols: (○), dry cell mass; open bar columns, the relative transcription and expression levels of *ppc*/*gap* as normalized with respect to the initial transcription and expression levels at zero fermentation time. The numbers in the columns represent the ratios of the *ppc*/*gap* transcription and expression levels at varying fermentation time. The average values from triplicate experimental data are given and the error bars indicate standard deviation.

and the uncoupled cell growth (a low cell yield) of *Z. mobilis* ZM4.

Expression of Heterologous *fdh*1 and *bgl*A in *Z. mobilis* ZM4

Although the *gap* and *gfo* promoters used are relatively strong in *Z. mobilis* ZM4, relatively low expression levels of *fdh*1 and *bgl*A were observed (0.08 ± 0.01 U mg^{-1} for FDH and 0.022 ± 0.002 U mg^{-1} for BGLA) when compared with their expression levels in *E. coli* BL21 (DE3)/pET-28a system (101.2 U mg^{-1} for FDH and 5.35 U mg^{-1} for BGLA). This observation is consistent with the findings of others who have attempted to improve the expression level of cellulase genes in *Z. mobilis* strains (Linger et al., 2010). More studies are needed to reveal the gene regulation and expression mechanisms of these heterologous genes in *Z. mobilis*.

Transformation Efficiencies

The newly constructed plasmids were transformed into *Z. mobilis* ZM4 and/or *Z. mobilis* ZM6 and their transformation efficiencies were compared to those of other plasmids transformed into *Z. mobilis* ZM4 as shown in Table III.

The results show that pHW20a can be most efficiently transconjugated in *Z. mobilis* ZM6 ($7.3–8.5 \times 10^{-5}$) and *Z. mobilis* ZM4 ($3.4–5.6 \times 10^{-5}$) as compared to other plasmids evaluated under the same experimental conditions. Under the same experimental conditions used in this experiment, the transformation efficiency of pHW20a is approximately two to three orders of magnitude greater than that of pLOI193 (13.4 kb, $8.2–20.1 \times 10^{-8}$) and about three orders of magnitude greater than that of pBBR1MCS-2-*tc*^{r} (5.2 kb, $1.5–2.0 \times 10^{-8}$). The transconjugation efficiency of pLOI193 in *Z. mobilis* ZM4 is significantly lower than that reported by Conway ($\sim 10^{-2}$) (Conway et al., 1987a). Other variables involved in transconjugation process are complex and the optimal conditions for higher transformation efficiency need be further studied.

A trend of gradually improved transformation efficiencies is observed with decreasing plasmid size when these plasmids harbor two *mob* genes, except for pHW20a-*mdh* (11.5 kb, $\sim 10^{-10}$) (Table III). When *sm*^{r}A, *sm*^{r}B, *cm*^{r}, and ColE1 were deleted from pLOI193 (13.4 kb, $\sim 10^{-7}$), a significant improvement of transformation efficiency of pHW10a (9.5 kb, $\sim 10^{-4}$) was observed. An improved transconjugation efficiency of pHW20a-SSC (12.0 kb, $\sim 10^{-6}$) is also observed when the plasmid size is reduced by 1.4 kb. When two plasmids of similar sizes, pLOI193 (13.4 kb) having ColE1 and pLOI204 without ColE1 (13.2 kb), are compared, similar transformation efficiency

Table III. Transformation efficiency of recombinant *Z. mobilis* strains by transconjugation.

Plasmid	Recipient cell	Plasmid size (kb)	Transformation efficiency	Specific activity (U mg^{-1})
pHW10a	*Z. mobilis* ZM4	9.5	$7.1–9.6 \times 10^{-5}$	—
pHW20a	*Z. mobilis* ZM4	9.9	$3.4–5.6 \times 10^{-5}$	3.2 ± 0.07 (GFOR)
pHW20a-*fdh*	*Z. mobilis* ZM4	11.1	$0.9–9.3 \times 10^{-6}$	0.08 ± 0.01[b]
pHW20a-*gfo*	*Z. mobilis* ZM4	11.5	$5.5–6.9 \times 10^{-5}$	6.80 ± 0.15
pHW20a-*bgl*A	*Z. mobilis* ZM4	11.7	$1.4–2.0 \times 10^{-6}$	0.022 ± 0.002[b]
pHW10a-SSC	*Z. mobilis* ZM4	12.0	$0.5–1.7 \times 10^{-6}$	—
pHW20a-*ppc*	*Z. mobilis* ZM4	13.0	$0.2–0.6 \times 10^{-7}$	Undetectable
pHW20a-*mdh*	*Z. mobilis* ZM4	11.5	$\sim 1.7 \times 10^{-10}$	0.07 ± 0.002
pLOI193	*Z. mobilis* ZM4	13.4	$0.8–2.0 \times 10^{-7}$	—
pBBR1MCS-2-*tc* [a]	*Z. mobilis* ZM4	5.2	$1.5–2.0 \times 10^{-8}$	—
pHW20a	*Z. mobilis* ZM6	9.9	$7.3–8.5 \times 10^{-5}$	—
pHW20a-*gfo*	*Z. mobilis* ZM6	11.5	$9.3–10.1 \times 10^{-5}$	5.05 ± 0.10

[a]pBBR1MCS-2-*tc*^{r} only has one *mob* gene and other plasmids have two *mob* genes originated from RP4 and RSF1010, respectively.
[b]Enzymatic activities were detected with the recombinant cells cultivated in YPMAG20 medium (containing 20 g L^{-1} glucose) and collected at late exponential phase ($OD_{600nm} = 2.0$). Fill in GFOR cellular activity: 394 ± 0.07 U/g-DCM for pHW20a and 837 ± 66 U/g-DCM for pHW20a-*gfo*.

($\sim 10^{-7}$) was observed for both plasmids (Conway et al., 1987a). This result suggests that the ColE1 deletion did not affect the transformation efficiency despite a small change of the plasmid size. On the other hand, a small mobilizable pBBR1MCS-2-*tc*^{r} (5.2 kb) derived from pBBR1MCS-2, which had been transformed into *Z. mobilis* by electroporation (Jeon et al., 2005), showed significantly lower transformation efficiency ($\sim 10^{-8}$) compared to that ($\sim 10^{-5}$) of pHW20a (9.9 kb), indicating that a significantly higher transconjugation efficiency obtained is attributable to two *mob* genes of pHW20a, as confirmed by others (Conway et al., 1987a).

Relatively high transformation efficiency for *Z. mobilis* (pHW20a-*gfo*) having an amplified homologous *gfo* gene was observed ($\sim 10^{-5}$). On the other hand, the transformation efficiency of *Z. mobilis* (pHW20a-*mdh*) containing a heterologous *mdh* gene from *E. coli* was found to be very low ($\sim 10^{-10}$). The plasmids containing heterologous genes, pHW20a-*fdh*, pHW20a-*bgl*A, and pHW10a-SSC, all showed improved transformation efficiency ($\sim 10^{-6}$). The significantly lower transformation efficiency observed in pHW20a-*mdh* compared to that of pHW20a-*gfo*, although these two plasmids are similar in size, is possibly due to incompatibility or competition between the endogenous "incomplete TCA cycle" linked metabolic pathways and the heterologous *mdh* expression linked anaplerotic reaction pathway in *Z. mobilis* ZM4 (pHW20a-*mdh*) recombinant strain. Further studies are needed to learn more about the effect of these competing metabolic pathways on transformation efficiency in the *Z. mobilis* ZM4 recombinant strain.

Stabilities of the New Plasmids Constructed

The results of stabilities studied for plasmids in *Z. mobilis* ZM4 host recombinant are shown in Figure 5. The newly designed pHW20a with an *ori*V replicon was very stable in host recombinant *Z. mobilis* ZM4 without any antibiotics. The plasmids containing *ori*V replicon, pLOI197, pLOI193, and pLOI204, are reportedly stable (Conway et al., 1987a). The relatively good stabilities of pHW20a and other pHW20a-derived plasmids reported in this work could be explained in part by the relaxed replication control of *ori*V, which is independent of the chromosome replication in the host cells (Paulsson and Ehrenberg, 1998). For those new plasmids, pHW20a, pHW20a-*fdh*, pHW20a-*bgl*A, and pHW20a-*mdh*, the fraction of the plasmid harboring cells in the total cell population remained at high levels (about 70–90%) throughout 120 generation times without *Tc* selection pressure. However, relatively low plasmid stability was observed for the pHW20a-*gfo*. The fraction of plasmid harboring cells of *Z. mobilis* ZM4 (pHW20a-*gfo*) gradually decreased and reached practically nil after about 50 generation times. The low expression levels of *fdh*1, *bgl*A, and *mdh*, as determined by the enzyme activity, did not cause metabolic stress on the host cells and plasmids were very stable. In contrast, the relatively lower stability of pHW20a-*gfo* is most likely attributable to the metabolic stress caused by the over-expression of amplified *gfo* gene. Our results confirmed the well-established relationship between the plasmid stability and metabolic stress of many plasmid containing recombinants (Diaz Ricci and Hernández, 2000; Ow et al., 2006).

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