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Research Article

## Three new shuttle vectors for heterologous expression in *Zymomonas mobilis*



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## ABSTRACT

**Background:** *Zymomonas mobilis*, as a novel platform for bio-ethanol production, has been attracted more attention and it is very important to construct vectors for the efficient expression of foreign genes in this bacterium.

**Results:** Three shuttle vectors (pSUZM1, pSUZM2 and pSUZM3) were first constructed with the origins of replication from the chromosome and two native plasmids (pZMZ401 and pZMZ402) of *Z. mobilis* ZM4, respectively. The three shuttle vectors were stable in *Z. mobilis* ZM4 and have 3, 32 and 27 copies, respectively. The promoter Ppdc (a), from the pyruvate decarboxylase gene, was cloned into the shuttle vectors, generating the expression vectors pSUZM1(2, 3)a. The codon-optimized glucoamylase gene from *Aspergillus awamori* combined with the signal peptide sequence from the alkaline phosphatase gene of *Z. mobilis* was cloned into pSUZM1(2, 3)a, resulting in the plasmids pSUZM1a-GA, pSUZM2a-GA and pSUZM3a-GA, respectively. After transforming these plasmids into *Z. mobilis* ZM4, the host was endowed with glucoamylase activity for starch hydrolysis. Both pSUZM2a-GA and pSUZM3a-GA were more efficient at producing glucoamylase than pSUZM1a-GA.

**Conclusions:** These results indicated that these expression vectors are useful tools for gene expression in *Z. mobilis* and this could provide a solid foundation for further studies of heterologous gene expression in *Z. mobilis*.

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### 1. Introduction

Biofuel ethanol may be used as a clean energy source to solve the problem of the forthcoming oil shortage. Biofuels can be produced by ethanologenic microorganisms, such as *Zymomonas mobilis*, which has been widely investigated for its effective production of ethanol with higher ethanol yields and tolerance, lower biomass and faster sugar uptake rate [1,2]. Expanding the spectrum of fermentable substrates and reducing the by-products are the main goals of the research. Genetic engineering approaches in *Z. mobilis* have commonly utilized plasmid vectors to express endogenous and/or heterologous genes to improve strains [3]. Thus, a highly efficient gene expression system is an important tool for genetic engineering of *Z. mobilis*.

Construction of efficient expression vectors is a key requirement for engineering *Z. mobilis*. In *Z. mobilis*, routine cloning vectors derived from pBR322 or pUC18 cannot be stably-maintained [4]. On the other hand, broad host-range vectors pBBR1MCS and RSF1010 have been successfully used for heterologous gene expression in *Z. mobilis* [4]. The vector pBBR1MCS2 with the promoter of the pyruvate decarboxylase gene (Ppdc) was employed to construct a

novel cell-surface display system for heterologous gene expression in *Z. mobilis* [5]. The vector RSF1010 containing the tac promoter and the lacI<sup>q</sup> repressor gene was used to express *Z. mobilis* genes encoding alcohol dehydrogenase I (*adhA*), alcohol dehydrogenase H (*adhB*), and pyruvate decarboxylase (*pdh*) [6]. However, these broad host-range vectors are unstable and their relatively large size limits their uses [4]. Shuttle vectors incorporating replicons from *Escherichia coli* plasmids and replication-related segments of native plasmids from *Z. mobilis* strains have been most commonly used for gene expression in *Z. mobilis* [7,8]. Four native plasmids pZMO1, pZMO2, pZMO3 and pZM3 of *Z. mobilis* ATCC 10988 were analyzed and used for the construction of shuttle vectors [4]. The native plasmids from two other *Z. mobilis* strains (ZM6100 and NCIMB 8827) were also used for the same purpose [9,10]. The shuttle vectors including genes related to carbohydrate or protein utilization by the bacteria were useful to broaden the substrate range of the *Z. mobilis* strain [11]. The results also indicated that the shuttle vectors were stably maintained in multiple copies per cell in *Z. mobilis* [4].

*Z. mobilis* ZM4 has a 2.06 Mb genome [12,13] and contains five native plasmids (pZMZ401–405) with a total length of 138 kb (<http://www.ncbi.nlm.nih.gov>). The unique replication origin locus, known as *oriC*, is present on the chromosome of all eubacteria and varies in size between 100 to 1000 bp [14]. The *oriC* of *Z. mobilis* ZM4 is located in a 656 bp region around 0 kb on the chromosome [15]. The plasmids

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pZM401 and pZM402 are 37.0 kb and 33.9 kb in size, and their replicase genes are 1.7 kb and 1.5 kb, respectively. Both the *oriC* of the *Z. mobilis* ZM4 chromosome and replication-related segments of native plasmids could provide a basis for constructing the autonomously replicating vectors for *Z. mobilis*. Since a strong promoter is needed to ensure the expression of heterologous genes, the promoter of the pyruvate decarboxylase gene, which is a constitutively expressed gene, was used to ensure the abundant expression of protein in *Z. mobilis* [16].

In this study, we constructed shuttle vectors for *Z. mobilis* with the *oriC* of *Z. mobilis* ZM4 chromosome and replication-related segments of native plasmids. The constitutive promoter *P<sub>pdC</sub>* was cloned into above shuttle vectors to generate the expression vectors, which were used to express a codon-optimized glucoamylase gene from *Aspergillus awamori* fused to the alkaline phosphatase signal peptide sequence from *Z. mobilis*. The stability and the expression efficiency of the three shuttle vectors were investigated in *Z. mobilis* ZM4.

## 2. Materials and methods

### 2.1. Bacterial strains, culture conditions and plasmids

*Z. mobilis* ZM4 and ATCC 29191 used throughout this study were purchased from the American Type Culture Collection (ATCC), while *Z. mobilis* CICC10232 and CICC 10225 were purchased from the China Center of Industrial Culture Collection (CICC) [17]. *E. coli* DH5 $\alpha$  were used for plasmid construction and amplification. During culture, 50  $\mu$ g/mL of kanamycin was added to LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, and plates with 1.5% Bacto agar) for *E. coli*, and 200  $\mu$ g/mL of kanamycin was added to RMG (Rich medium with glucose) medium (1% yeast extract, 0.2% KH<sub>2</sub>PO<sub>4</sub>, 2% glucose, and plates with 1.8% Bacto agar) for *Z. mobilis* [18].

The vectors pBBR1MCS-2 and pUC18 provided the kanamycin-resistance gene and *ori*, respectively, for constructing shuttle vectors for *Z. mobilis* [19]. The glucoamylase gene (GA) of *A. awamori* was codon optimized and synthesized (TaKaRa, Dalian, China).

### 2.2. Plasmids construction

All DNA manipulations were performed according to standard protocols [18]. The primers used in this study are shown in Table 1. The PCR condition was as follows: pre-denaturation at 94°C for 2 min, 30 cycles of denaturation at 98°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 2 kb/min, and a final 10 min extension at 72°C. PCR products were gel-purified, treated with T4 DNA polymerase (TaKaRa, Dalian, China) to generate ssDNA ends, annealed to homologous stretches, and recombined together. The vectors were constructed by using the method called sequence and ligation-independent cloning (SLIC) [20,21].

The primers (either KanCF, KanP1F or KanP2F with KanR) (Table 1) were used to amplify the kanamycin resistance gene from pBBR1MCS-2. The primers (OriEF with either OriECR, OriEP1R or OriEP2R) (Table 1) were used to amplify the replication region of pUC18. The primers OriZCF/OriZCR, OriZP1F/OriZP1R and OriZP2F/OriZP2R (Table 1) were used to amplify the *oriC* from the *Z. mobilis* ZM4 chromosome, the replication related region from the native plasmid pZM401 and pZM402, respectively. The shuttle vectors were constructed using SLIC [20], resulting in the plasmid pSUZM1, pSUZM2 and pSUZM3, respectively.

The primers (P<sub>pdC</sub>F with either P1<sub>pdC</sub>R, P2<sub>pdC</sub>R or P3<sub>pdC</sub>R) (Table 1) for the amplification of the vector DNA fragment were designed based on the sequence of pSUZM1, pSUZM2 and pSUZM3. The primers (either 1<sub>pdC</sub>F, 2<sub>pdC</sub>F or 3<sub>pdC</sub>F with pdC R) (Table 1) were used to amplify the promoter of pyruvate decarboxylase gene from *Z. mobilis* ZM4 genomic DNA. The expression vectors were constructed using SLIC [20], resulting in the plasmid pSUZM1a, pSUZM2a and pSUZM3a, respectively.

The primers VGAF/VGAR (Table 1) for the amplification of the vector DNA fragment were designed based on the pSUZM1a (pSUZM2a, pSUZM3a) sequence. The primers GAF/GAR (Table 1) for the amplification of the GA gene were designed based on the corresponding nucleotide sequences of GA gene from plasmid pUC-GA. The GA expression plasmid was constructed using SLIC [20], resulting in the plasmid pSUZM1a-GA, pSUZM2a-GA and pSUZM3a-GA, respectively.

### 2.3. Z. mobilis transformation

Plasmid DNA was transformed into *Z. mobilis* ZM4 by electroporation [16]. For *Z. mobilis*, DNA was transformed into cells suspended in 200  $\mu$ L of glycerol buffer [16]. A Bio-Rad Gene Pulser was used with the following conditions: 2.5 kV and 10 ms in a 0.2 cm cuvette. After electroporation, the cells were added to 3 mL of RMG medium and incubated at 30°C for 16 h without agitation. All of the recovered cells were plated onto RMG agar plates containing 200  $\mu$ g/mL of kanamycin and incubated at 30°C for 2 or 3 d. Positive transformants were identified by PCR.

### 2.4. Plasmid stability

Plasmid stability assay was carried out as described by So with minor modifications [4]. Freshly transformed *Z. mobilis* strains were grown at 30°C for 24 h without agitation in RMG medium containing 200  $\mu$ g/mL of kanamycin. Aliquots were diluted 1:100 in fresh RMG media without kanamycin (10 mL), and were cultured at 30°C for 24 h without agitation. This iterative subculturing process was repeated every 24 h for 10 consecutive days. Aliquots were taken every 24 h and were plated on RMG plates, and checked for the presence of the plasmid by replica plating on RMG plates containing kanamycin.

**Table 1**  
Primers used for construction of recombinant plasmids.

Primer	Sequence (5'–3')
KanCF	GGTAGAAGCCGCGACTGAAGGTAGCTTGCAGTGGG
KanP1F	CGTAATCTCGCGTTAATCGAGGTAGCTTGCAGTGGG
KanP2F KanR	CTTCTGTCGCGCTCAGAGGTAGCTTGCAGTGGG GGATCTTCACTAGATCTCGATTCCGAAGCCC
OriEF	GGGCTTCGGAATCGAGGATCTAGGTGAAGATCC
OriECR	CTTACCCTGGTTCGAAGAGGAAACCTGTCGTGCC
OriEP1R	GCATGTCCTTTCGCGTGCAGAAACCTGTCGTGCC
OriEP2R	CAGGATACCTTTCGCGGAAACCTGTCGTGCC
OriZCF	GGCAGCACAGGTTTCTCTTTCGACCAGCGGTAAG
OriZCR	CCCCTGCAAGCTACCTTCAGTCCGGCTTCTTACC
OriZP1F	GGCAGCACAGGTTTCCGCACCCGAAAGGACATG
OriZP1R	CCCCTGCAAGCTACCTCGATTAACCCGAGGATTACC
OriZP2F	GGCAGCACAGGTTTCCCGAAAGGTCATCCTG
OriZP2R	CCCCTGCAAGCTACCTTCGAGGGACGACAG
P <sub>pdC</sub> F	ATATATGGAGTAAGCAAGGTAGCTTGCAGTGGG
P1 <sub>pdC</sub> R	GCGATCATGAGCGTAATCAGTCGCGGCTTCTACC
P2 <sub>pdC</sub> R	GCGATCATGAGCGTAACGATTAACCCGAGGATT
P3 <sub>pdC</sub> R	GCGATCATGAGCGTAACTGAGGGACGACAGAAGC
1 <sub>pdC</sub> F	GGTAGAAGCCGCGACTGATTACGCTCATGATCCG
2 <sub>pdC</sub> F	AATCTGCGGTTAATCGTTACGCTCATGATCCG
3 <sub>pdC</sub> F	GCTTCTGTCGTCGCTCAGTTACGCTCATGATCCG
pdC R	CCCCTGCAAGCTACCTTCTTACTCCATATAT
V-GAF V-GAR	GTTACCAGTACCTGGCGTTAAAGGTAGCTTGCAGTGGG GATGCAGCAGGGAGTTCAITGCTTACTCCATATAT
GAF GAR	ATATATGGAGTAAGCAATGAACCTCCCTGCTCATCA CCCCTGCAAGCTACCTATCATCTTCCAGGGGGTAC

### 2.5. Determination of plasmid copy number by quantitative real time PCR (qPCR)

The sequences of chromosome and the native plasmids of *Z. mobilis* ZM4 were obtained from the National Center for Biotechnology Information (NCBI) public database. PCR primers specific for the vectors and the chromosome of *Z. mobilis* ZM4 were designed to determine the number of plasmid molecules per chromosome molecule in each sample using specific primer sets (Table 2). The gene *psc* (ZMO1360), encoding pyruvate decarboxylase on the *Z. mobilis* ZM4 chromosome was chosen as a single copy reference gene. The kanamycin resistance gene from each shuttle plasmid was chosen for pSUZM1, pSUZM2 and pSUZM3 plasmid copy number determination. The *Z. mobilis* transformants were incubated without agitation at 30°C to OD<sub>600nm</sub> of 0.25 in RMG media with 200 µg/mL of kanamycin. The freshly-inoculated cultures were heated at 95°C for 10 min followed by immediate freezing at -20°C and used as the templates for quantitative (real time) PCR analyses [4]. qPCR was performed using an iQ5 Real-time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with reaction mixtures (total volume 20 µL) containing 10 µL 2xTaKaRa SYBR Green Real-Time PCR Master Mix (Takara, Dalian, China), 1.5 µL forward and reverse primers (10 µM stock) and 1 µL of sample. The PCR condition was as follows: 98°C for 2 min, 40 cycles of 98°C for 10 s, 50°C for 10 s, and 68°C for 30 s, followed by a gradient temperature from 55°C to 95°C, the resulting melting curves were used to confirm the specificity of the primer pairs in each experiment [22]. All qPCR experiments were performed with three independent biological replicates. The plasmid copy numbers were determined using the equations of plasmid copy number determination to calculate [23].

### 2.6. Native-PAGE

One mL of the *Z. mobilis* transformant culture (OD<sub>600</sub> = 2.0) was centrifuged at 4000 rpm for 10 min and the pellet was suspended into 500 µL PBS buffer and was sonicated, with 20 µL of either the intracellular or extracellular fractions used for PAGE. For 8% Native-PAGE (containing 1 mL 5% soluble starch in separation gel), each sample was mixed with 5xNative loading buffer (250 mmol/L Tris-HCl (pH 6.8), 50% Glycerol, 0.5% Bromophenol blue). After electrophoresis, the Native-PAGE gel was incubated at 37°C for 4–5 h in Lugol's iodine for activity staining and was also stained with Coomassie Brilliant Blue.

### 2.7. Glucoamylase assay

Three *Z. mobilis* transformant cultures (0.5 mL each, OD<sub>600</sub> = 2.0) were centrifuged at 5000 rpm, 4°C for 10 min and the cell pellets were re-suspended in 0.5 mL ddH<sub>2</sub>O, followed by sonication to release intracellular glucoamylase. Glucoamylase activity was assayed by mixing 0.1 mL sample, 2.5 mL 2% gelatinized starch and 10 mM McIlvaine buffer (pH 3.8) and incubating for 10 min at 50°C. The reaction was stopped by incubation for 10 min at 100°C. The enzyme digest was added to 2 mL DNS solution (3, 5-dinitrosalicylic acid reagent: 3, 5-dinitrosalicylic acid 5 g, phenol 1 g, sodium sulfite 0.25 g, sodium potassium tartrate 100 g dissolved in 250 mL of 0.5 M NaOH and diluted to 500 mL with water), and the tube was placed in a

boiling water bath for 5 min and put on ice again. After the addition of 1 mL H<sub>2</sub>O, the amount of reducing sugar was determined by reading the absorption at 540 nm [24]. A series of glucose concentration standards were included with each reducing sugar assay. One enzymatic unit was defined as the amount of enzyme capable of producing 1 mg reducing sugar under the conditions described above.

### 2.8. Fermentation conditions and analytical methods

Fermentation and analytical methods were performed according to Wang et al [16]. *Z. mobilis* ZM4 harboring pSUZM1a was used as a control. The four *Z. mobilis* transformants mentioned above were grown in RMG medium containing kanamycin (200 µg/mL) without shaking for 24 h at 30°C. Twenty-five milliliters of culture were transferred into 0.5 L medium containing soluble starch (5% soluble starch, 0.5% glucose, 1% yeast extract and 0.2% KH<sub>2</sub>PO<sub>4</sub> pH 6.0) and incubated with gentle agitation at 30°C. Samples were taken every 12 h for the determination of cell growth, ethanol production and reducing sugar. Triplicate fermentations were performed for each strain.

Samples were taken from each fermentation every 12 h for the following analyses. Cell growth was determined by measuring optical density (OD) at 600 nm. Ethanol was assayed using GC103 with a glass column (0.26 × 200 cm) filled with Porapak type QS (80–100 mesh, Waters, Milford, MA) at 150°C and a FID detector at 80°C. Molecular nitrogen (N<sub>2</sub>) was the carrier gas (30 mL/min). Reducing sugars were determined according to the DNS method [25].

## 3. Results

### 3.1. Construction of vectors and glucoamylase-containing plasmids

The shuttle vectors pSUZM1, pSUZM2 and pSUZM3 were constructed as shown in Fig. 1. Firstly, the kanamycin resistance gene of pBBR1MCS-2 and the replication region of pUC18 were amplified by PCR, yielding 1.1 kb and 0.95 kb fragments, respectively. *OriC* from the *Z. mobilis* ZM4 chromosome and the replication-related segments of plasmid pZM401 and pZM402 were amplified by PCR to yield 1.1 kb, 2.8 kb and 2.4 kb products, respectively. These products were used in SLIC reactions with the kanamycin resistance gene and replicon of pUC18, to give the shuttle vectors pSUZM1, pSUZM2 and pSUZM3 (Fig. 1).

The constitutive *Ppdc* promoter of *Z. mobilis* ZM4 was previously shown to strongly express foreign genes [16] and was used to construct the expression vectors pSUZM1a, pSUZM2a and pSUZM3a (Fig. 1).

In order to express the codon-optimized glucoamylase gene of *A. awamori* with the signal peptide sequence from the *Z. mobilis* alkaline phosphatase gene, the recombinant plasmids pSUZM1a-GA, pSUZM2a-GA and pSUZM3a-GA were constructed (Fig. 1). All above vectors and plasmids were verified by using PCR, restriction enzyme analyses and DNA sequencing analysis (BGI, Shenzhen, China).

### 3.2. Stability of shuttle vectors in different *Z. mobilis* strains

A phylogenetic tree of four *Z. mobilis* strains stored in our laboratory was constructed by using CVtree (<http://tlife.fudan.edu.cn/cvtree>) [26] with a K value of 6. The genome sequence of *Z. mobilis* ZM4 (GenBank accession no. 283856168) and ATCC 29191 (GenBank accession no. 397675890) were downloaded from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), while those of *Z. mobilis* CICC 10232 and CICC 10225 were sequenced by our laboratory (unpublished data). The result showed that strains CICC 10225 and CICC 10232 were very close to each other and clustered closely to ZM4. The ATCC 29191 strain had a more distant relationship to other three strains (Fig. 2).

The segregational stability of pSUZM1, pSUZM2 and pSUZM3 was tested in the four strains mentioned above. The stability of the three

**Table 2**

Primers used for the quantitative real time PCR analysis.

Primer	Sequence (5'–3')	Description
KanqF	CTGATGCCGCCGTGTT	Specific primers for kanamycin resistance gene
KanqR	AGCAGCCAGTCCCTTCC	
pdcqF	CAAAGTTGCCGCTCTC	Specific primers for pyruvate decarboxylase gene
pdcqR	GGTGCCGATGTAATGC	

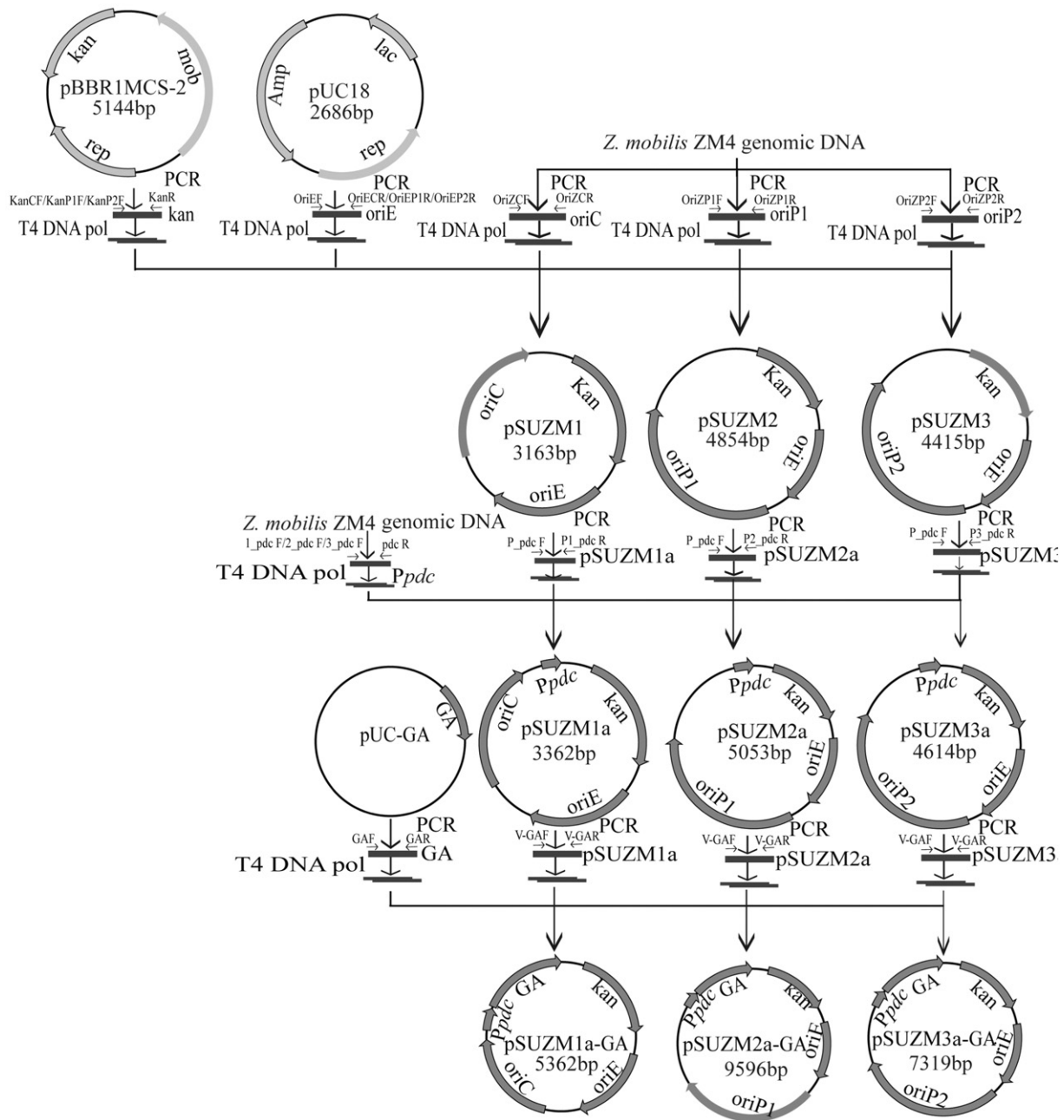


Fig. 1. Schematic diagram outlining the construction of the plasmids used in this study. The primers used in this procedure are briefly shown.

shuttle vectors in *Z. mobilis* ZM4 was stronger than that in the other three strains (Fig. 3). In the strains CICC 10232 and CICC 10225, both pSUZM2 and pSUZM3 were much more stable than pSUZM1 (Fig. 3). However, in *Z. mobilis* ATCC 29191, all three vectors exhibited poor stability and the number of Kan<sup>r</sup> colonies was close to zero after 10 d of kanamycin-free incubation (Fig. 3).

### 3.3. Copy number of three shuttle vectors in *Z. mobilis* ZM4

In addition to stability, the expression efficiency is usually related to the copy number of the vector. qPCR can be used to determine the copy number of a particular plasmid [23]. We designed two sets of specific qPCR primers for genes residing on the chromosome (*pdc*) or the vectors (kanamycin-resistance gene). The copy number of pSUZM2 and pSUZM3 was 27 and 32, respectively, which is notably higher than that of pSUZM1, at only 3 copies per cell (Fig. 4).

### 3.4. Expression and activity of glucoamylase gene in *Z. mobilis*

The utility of the vectors for heterologous gene expression was determined by expressing a codon-optimized version of the *A. awamori* glucoamylase gene fused to the signal peptide sequence of *Z. mobilis* alkaline phosphatase. The glucoamylase gene-containing plasmids pSUZM1a-GA, pSUZM2a-GA and pSUZM3a-GA, as well as the expression vectors pSUZM1a, pSUZM2a, and pSUZM3a, were transformed into *Z. mobilis* ZM4, respectively. The extracellular and intracellular proteins were extracted from transformed cells and used for native-PAGE analysis. The glucoamylase activity of all transformants harboring the GA-expression plasmids was detected in both intracellular and extracellular fractions by Coomassie and activity staining (Fig. 5a).

The results of the glucoamylase activity assay showed marked differences among the three kinds of transformants:

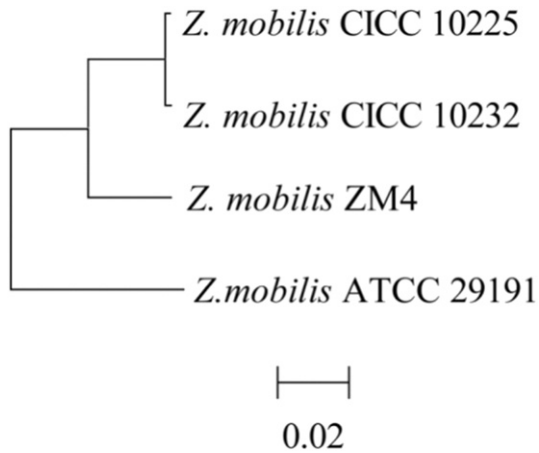


Fig. 2. The phylogenetic tree analysis of the four *Z. mobilis* strains.

pSUZM2a-GA-transformed strain exhibited the highest glucoamylase activity (47.10 U/L) and the highest percentage of activity (73.3%) in the extracellular fraction compared to total activity. The pSUZM1a-GA-containing strain had the lowest glucoamylase activity (5.21 U/L), while the percentage of activity was 60.3%. The

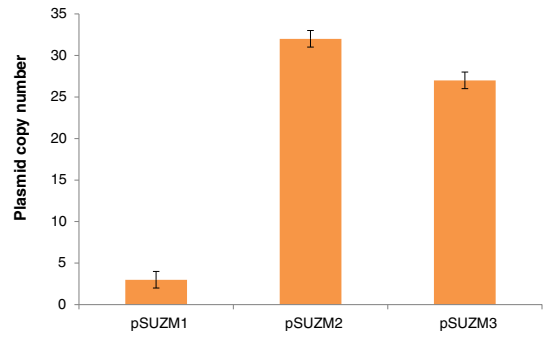


Fig. 4. Quantitative PCR analysis of the plasmid copy number of pSUZM1, pSUZM2 and pSUZM3 in *Z. mobilis* ZM4.

strain containing pSUZM3a-GA had glucoamylase activity of 43.13 U/L and the percentage of activity was 59.1% (Fig. 5b).

### 3.5. Fermentation

Since a small amount of glucose is essential to start the fermentation of *Z. mobilis*, 0.5% glucose was added to the fermentation

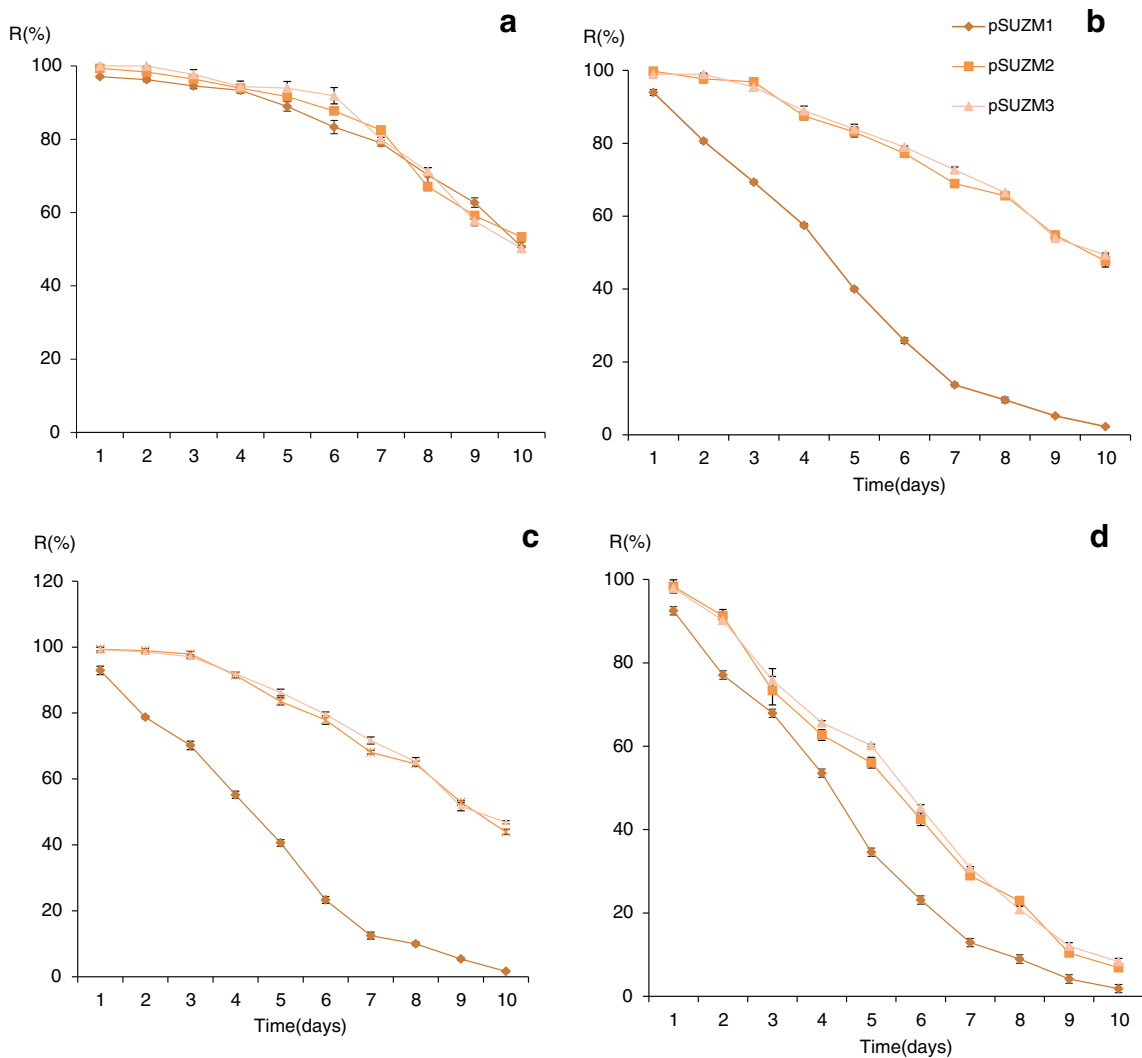
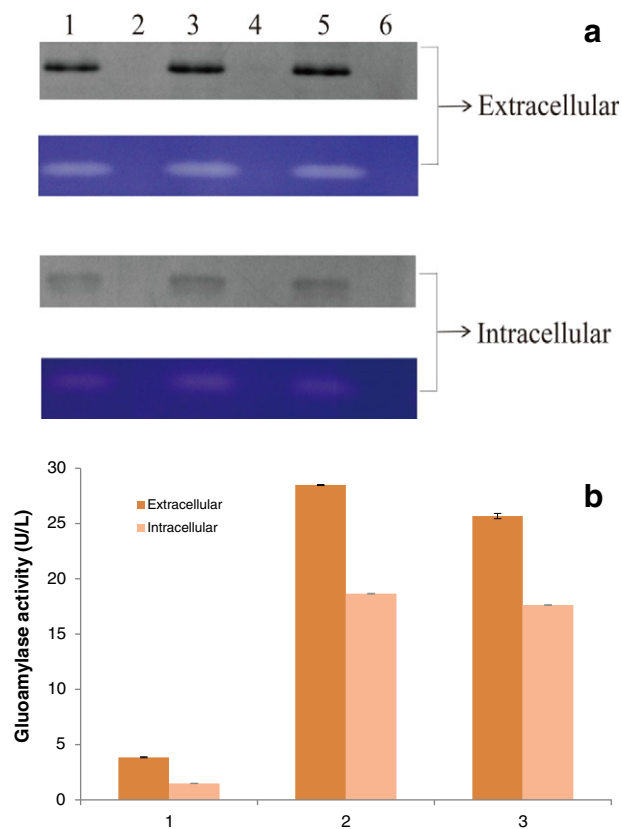


Fig. 3. Segregational stability of the three shuttle vectors in different *Z. mobilis* strains. R: the ratio of the number of the colonies which could grow on kanamycin-containing RMG plates to the number of the total checking colonies. (a) *Z. mobilis* ZM4; (b) *Z. mobilis* CICC 10225; (c) *Z. mobilis* CICC 10232; (d) *Z. mobilis* ATCC 29191.



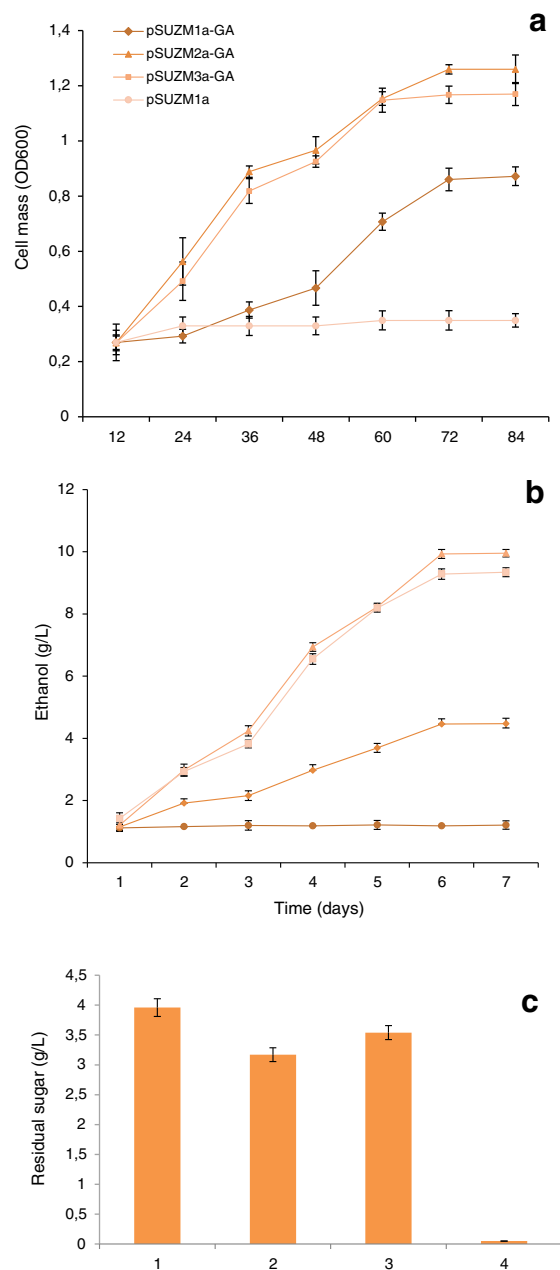
**Fig. 5.** Expression and activity assay of glucoamylase in *Z. mobilis* transformants. (a) Native-PAGE of the intracellular and extracellular glucoamylase of recombinant *Z. mobilis*. Upper panel: Extracellular 1, 3, 5: pSUZM1(2,3)a-GA; 2, 4, 6: pSUZM1(2,3)a; Lower panel: Intracellular 1, 3, 5: pSUZM1(2,3)a-GA; 2, 4, 6: pSUZM1(2,3)a; Gray gel images: Coomassie staining; Blue gel images: activity staining. (b) Glucoamylase activity of the intracellular and extracellular fraction of the *Z. mobilis* recombinants. 1: pSUZM1a-GA; 2: pSUZM2a-GA; 3: pSUZM3a-GA.

medium [16]. When the three strains containing the GA-expression plasmids were used for ethanol fermentation from soluble starch it was found that there were obvious differences in the biomass of each strain. The pSUZM2a-GA- and pSUZM3a-GA-containing cells exhibited the highest levels of cell growth, with the OD<sub>600</sub> values of 1.26 and 1.17, respectively, whereas the pSUZM1a-GA containing strain showed relatively poor growth with an OD<sub>600</sub> of 0.87 (Fig. 6a).

Cells containing the pSUZM2a-GA plasmid had the highest levels of ethanol production with 9.95 g/L and the ethanol was continually produced until about the 6th day of fermentation, which was nearly 35.02% of the theoretical yield of ethanol from soluble starch (Fig. 6b). Reducing sugar content of the pSUZM2a-GA-containing strain at the end of fermentation was the lowest at only 3.17 g/L (Fig. 6c). The pSUZM1a-GA- and pSUZM3a-GA-transformed cells produced 4.47 g/L and 9.34 g/L ethanol, respectively, with 3.96 g/L and 3.54 g/L reducing sugars remained unconverted, and consequently ethanol yields were calculated to be 15.7% and 32.9% of theoretical value. The pSUZM2a-GA-harboring strain had the highest glucoamylase activity among the three strains resulting in the lowest reducing sugar content and the highest ethanol yield.

#### 4. Discussion

Construction of efficient expression vectors is a key requirement for engineering *Z. mobilis*. In *Z. mobilis*, routine cloning vectors derived from pBR322 or pUC18 are unable to be stably-maintained and the broad-host range plasmids derived from pBBR1MCS or



**Fig. 6.** Fermentation assay *Z. mobilis* recombinants. (a) Cell mass of the *Z. mobilis* recombinants. (b) Ethanol production from starch of the *Z. mobilis* recombinants. (c) Residual sugar of the *Z. mobilis* recombinants.

RSF1010 are also structurally unstable [20]. To the best of our knowledge, there are no reports of the use of replicons from *Z. mobilis* ZM4 for the construction of vectors. In this study the replication regions of the *Z. mobilis* ZM4 chromosome and two native plasmids from strain ZM4 were used to construct *E. coli*-*Z. mobilis* shuttle vectors.

The stability of vectors in different host strains depends on two factors. The first one is replication system of the vectors, while the other is related to the genetic background of host strains [4]. The stability of pSUZM1 in *Z. mobilis* ZM4 was higher than that in the other strains tested. The most likely reason for this is that the native ZM4 origin might be efficiently recognized by its own replication system. As the three shuttle vectors pSUZM1, pSUZM2 and pSUZM3 all possessed origins of replication from the chromosome or native plasmids of ZM4 they were all stable in the

ZM4 strain, as would be expected. The stability of the vectors pSUZM2 and pSUZM3 in CICC 10225, CICC 10232 and ZM4 were all higher than that in ATCC 29191, because these three strains are closely related genetically and replication of the two vectors depends on their own replicase genes and origins of replication. When pSUZM2 and pSUZM3 were transformed into *Z. mobilis* ZM4, they had the same replication-related segments as the native plasmids pZZM401 and pZZM402. As such they should all belong to the same incompatibility group and should not be able to coexist in the same cell. Previous studies demonstrated that there was direct competition between native pZMO2 and recombinant pZMO2-derived plasmids in the *Z. mobilis* CU1 Rif2 strain, native pZMO7 and recombinant pZMO7-derived plasmids in the *Z. mobilis* NCIMB11163 strain [4,8]. In the plasmid stability assay, only one plasmid of either pSUZM2 and pZZM401 or pSUZM3 and pZZM402 could exist in the daughter cells. Our results showed that there were still 50% of the cells that retained kanamycin resistance.

Overall, the stability of the shuttle vectors was associated with the genetic background of the host bacteria. According to the phylogenetic analysis of the host strains, a suitable vector should be chosen for a particular strain.

The copy numbers of the three shuttle vectors varied considerably in *Z. mobilis* ZM4 and were clearly related to the source of the replicons. The vector pSUZM1 containing the chromosomal *oriC* had a low copy number, which was directly related to the low copy number of the chromosome. Since the native plasmids pZZM401 and pZZM402 are high copy number plasmids, the vectors derived from these native plasmids (pSUZM2 and pSUZM3, respectively) showed similar copy numbers in the ZM4 strain. The copy numbers of the three shuttle vectors within the other three *Z. mobilis* strains were not detected. It was possible that the copy number of the shuttle vectors was different in each strain because their stability depended on the strains.

The utility of these expression vectors was determined using a codon-optimized glucoamylase gene from *A. awamori* with the signal peptide sequence of the alkaline phosphatase gene from *Z. mobilis*. Cells harboring the GA-expression plasmids with the replication-related segments of the native plasmids showed higher levels of activity than that containing *oriC* of the chromosome in the glucoamylase activity and ethanol production efficiency. The structure of the expression vector was a major reason for the different expression efficiencies, again due to the copy number of replicons from *Z. mobilis*.

Previous research in our lab has shown that a *Z. mobilis* ZM4 recombinant strain harboring the glucoamylase expression plasmids with the signal peptide sequence *zmo130* or *zmo331* of *Z. mobilis* had an ethanol production level of 3.8 g/L and reducing sugar content of 8.40 g/L (unpublished data). The fermentation capacity of this strain was lower than that of the pSUZM2a-GA and pSUZM3a-GA strains constructed in this study. The ethanol fermentation was performed with gentle shaking in this study to prevent *Z. mobilis* cells from settling down to the bottom of flask. The results could vary slightly to those under static conditions. The ethanol fermentation of above recombinant strains could be performed with raw starch which should be gelatinized by stirring and heating firstly. The cooking process might affect the ethanol titer and yield. These are worth further studying in our subsequent research.

The factors affecting ethanol production and the remained reducing sugar content included the promoter, the signal peptide and the kind of amylases [16]. Methods to improve ethanol production efficiency require the combined effect of several types of amylases instead of only glucoamylase [27], using different promoters, such as *Ptac* and *Para* [4,28], and optimizing fermentation conditions. The expression vectors reported here may provide valuable tools in research and genetic engineering of *Z. mobilis*.

## Conflicts of interest statement

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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