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A New Shuttle Plasmid That Stably Replicates in *Clostridium acetobutylicum*

Sang-Hyun Lee[1,2], Min-A Kwon[2], Sunhwa Choi[2], Sooah Kim[1], Jungyeon Kim[1], Yong-An Shin[2], and Kyoung Heon Kim[1]*

[1]*Department of Biotechnology, Graduate School, Korea University, Seoul 136-713, Republic of Korea*
[2]*R&D Center, GS Caltex Corporation, Daejeon 305-380, Republic of Korea*

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**Corresponding author*
Phone: +82-2-3290-3028;
Fax: +82-2-925-1970;
E-mail: khekim@korea.ac.kr

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We have developed a new shuttle plasmid, designated as pLK1-MCS that can replicate in both *Clostridium acetobutylicum* and *Escherichia coli*, by combining the pUB110 and pUC19 plasmids. Plasmid pLK1-MCS replicated more stably than previously reported plasmids containing either the pIM13 or the pAMβ1 replicon in the absence of antibiotic selective pressure. The transfer frequency of pLK1-MCS into *C. acetobutylicum* was similar to the transfer frequency of other shuttle plasmids. We complemented *C. acetobutylicum* ML1 (that does not produce solvents such as acetone, butanol, and ethanol owing to loss of the megaplasmid pSOL1 harboring the *adhE1-ctfAB-adc* operon) by introducing pLK1-MCS carrying the *adhE1-ctfAB-adc* operon into *C. acetobutylicum* ML1. The transformed cells were able to resume anaerobic solvent production, indicating that the new shuttle plasmid has the potential for practical use in microbial biotechnology.

Keywords: *Clostridium acetobutylicum*, shuttle plasmid, segregational stability

Introduction

The genus *Clostridium* consists of versatile, gram-positive, strictly anaerobic, and solvent-producing bacteria with extreme biocatalytic activities [5, 13, 15, 30]. However, the potential metabolic capacities of Clostridia have not been closely examined until recently, because genetic inaccessibility of these bacteria has prevented detailed research at the molecular level. The genome sequences of some Clostridia have been determined and this information provides new opportunities, including commercial production of biofuel from renewable biomass, elucidation of virulence mechanisms in pathogenic species, and development of protection methods for the species [5, 6, 24, 26, 31]. In spite of their physiological and industrial importance, research on the genus *Clostridium* is restricted owing to the absence of a proper system for genetic manipulation [9]. Accordingly, an effective host/plasmid system is needed to develop strains that have high metabolic activity.

Several shuttle plasmids that can replicate in both Clostridia and *E. coli* have been developed. Among them, plasmids containing the pIM13 and pAMβ1 replicons such as pIM1 and pMTL500E, respectively, replicate in *C. acetobutylicum*. However, none of these plasmids have been used for industrial applications because they are segregationally unstable in the absence of appropriate antibiotics during cultivation [20]. Therefore, it is necessary to develop a segregationally stable plasmid system for the industrial-scale production of biochemicals and biofuels by *C. acetobutylicum*.

In this study, we report that the new shuttle plasmid pLK1-MCS, which harbors the replication origins of both pUB110 and pUC19, can replicate in both *C. acetobutylicum* and *E. coli*. We also report that by using this shuttle plasmid, we can construct a recombinant plasmid that stably replicates and expresses recombinant genes in *C. acetobutylicum*.

Materials and Methods

Bacterial Strains, Plasmids, Primers, and Enzymes

The bacterial strains, plasmids, and primers used in this study

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristic	Reference
Strain		
C. acetobutylicum ATCC 824		
C. acetobutylicum ML1	pSOL1$^-$	This study
C. beijerinckii NCIBM 8052		
E. coli XL1-Blue		Stratagene
E. coli TOP10		Invitrogen
Plasmid		
pSM704	Neo^r, Ap^r, pUB110 origin, *repA*	[14]
pTHL1-Cm	pIM13 origin, Cm^r, Em^r, Ap^r, P_{Thl} promoter, *repL*	[10]
pMTL500E	pAMβ1 origin, Ap^r, Em^r	[21]
pGS1-MCS	pTHL1-Cm derivative with a substituted multiple cloning site, P_{Thl} promoter	This study
pAN1	Cm^r, Φ*3T I* gene, p15A origin	[18]
pLK1-MCS	Em^r, Ap^r, P_{Thl} promoter, pUB110 origin, *repA*	This study
pLK1-E1ABC	AdhE1$^+$, CtfAB$^+$, Adc$^+$	This study

are summarized in Tables 1 and 2. *E. coli* XL1-Blue was used as the cloning host, and *E. coli* TOP10 (pAN1) was used for *in vivo* methylation before transformation into *C. acetobutylicum*. Clostridia were used as host cells for the shuttle plasmids, and *C. acetobutylicum* ML1 was used for complementary expression of the *adhE1-ctfAB-adc* operon. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Pfu-X polymerase was purchased from Solgent (Daejeon, Korea).

Media and Culture Conditions

E. coli was grown aerobically in 2× YTG liquid medium (16 g Bactotryptone, 10 g yeast extract, 4 g NaCl, and 5 g glucose per liter) on a shaking incubator at 200 rpm, or on 2× YTG agar

Table 2. Primers used in this study.

Primer	[a]Sequence	Target
Primer 1	ATA**GGATCC**ACGAAGTCGAGATCAGGGAATGAG	pUB110 origin
Primer 2	GCG**AGATCT**AATCTTCAACAAACTGGCCCGTTTGTTGAA	
Primer 3	ATA**CCCGGG**CATGATTTTAAGGGGGTTAGCAGATGCATAAG	Thiolase promoter
Primer 4	CTC**GAGCTC**GGTGTGAAATACCGCACAGATGCG	
Primer 5	CAC**GTTTAAAC**ATGAAAGTCACAACAGTAAAGGAATTAGAT	*adhE1-ctfAB-adc* operon
Primer 6	ATA**GTCGAC**GAATCTATTATGCAGAATTTTTAGGAAGTGTAC	
Primer 7	CACGTTTAAACATGAAAGTCACAACAGTAAAGGAATTAGAT	*adhE1*
Primer 8	CACCTCGAGTTAAGGTTGTTTTTTAAAACAATTTATATACA	
Primer 9	CACGTTTAAACATGAAAGTTACAAATCAAAAAGAACTAAAAC	*adhE2*
Primer 10	CACTCTAGATTAAAATGATTTTATATAGATATCCTTAAG	
Primer 11	CACGTTTAAACATGAACTCTAAAATAATTAGATTTGAAAATTTAAG	*ctfAB*
Primer 12	CACGAATTCTTAAACAGCCATGGGTCTAAGTTCATTGGATATGA	
Primer 13	ATGTTAAAGGATGAAGTAATTAAACAAATTAGCAC	*adc*
Primer 14	TTACTTAAGATAATCATATATAACTTCAGCTCTAG	
Primer 15	AAACTTTAGTAAATAACGCTGGGTTTGGCGATTATAGCAA	pSOL1
Primer 16	GCTGTGTACCTTCAATTTTCTCATAGTCACGTACAAATA	
Primer 17	AGTAAAAGGGAGTGTACGACCAGTGATTAAGAGT	*ptb-buk*
Primer 18	ATGACTTTATTATTTGTATTCCTTAGCTTTTTCT	

[a]Restriction enzyme sites are shown in bold font.

medium at 30°C. All Clostridia were anaerobically cultured in clostridial growth medium (CGM) containing 0.75 g K_2HPO_4, 0.75 g KH_2PO_4, 0.7 g $MgSO_4 \cdot 7H_2O$, 0.017 g $MnSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 2 g $(NH_4)_2SO_4$, 1 g NaCl, 2 g asparagine, 0.004 g *p*-aminobenzoic acid, 5 g yeast extract, 4.08 g $CH_3COONa \cdot 3H_2O$, and 80 g glucose per liter. All media components used in this study were purchased from Difco (Detroit, MD, USA) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid Construction

For the construction of pLK1-MCS, PCR was performed to amplify the pUB110 origin using pSM704 as template DNA with primers 1 and 2. The resulting 1.3 kb DNA fragment was isolated using the Wizard SV Gel Clean-up System (Promega, Madison, WI, USA), digested with *Sac*I and *Bgl*II, and then subcloned into pMTL500E digested with *Sac*I and *Bam*HI to construct the plasmid pLK1-temp. Next, a DNA fragment containing the thiolase promoter derived from *C. acetobutylicum*, a ribosomal binding sequence (RBS; AGGAGG), and a unique multiple cloning site (*Pme*I, *Pst*I, *Bgl*II, *Xho*I, *Not*I, *Sal*I, *Xba*I, *Xma*I (*Sma*I), and *Eco*RI) was amplified with primers 3 and 4 using pGS1-MCS as template DNA. The resulting fragment was digested with *Sma*I and *Sac*I and subcloned into *Pvu*II- and *Sac*I-digested pLK1-temp to construct the plasmid pLK1-MCS (Fig. 1).

The *C. acetobutylicum adhE1-ctfAB-adc* operon, including the terminator of the *adc* gene, was amplified by PCR using the genomic DNA of *C. acetobutylicum* as a template and primers 5 and 6. The resulting 5.6 kb fragment was digested with *Pme*I and *Sal*I and then cloned into pLK1-MCS to make the plasmid pLK1-E1ABC. The genomic DNA for PCR was isolated using the Wizard Genomic DNA Purification Kit (Promega).

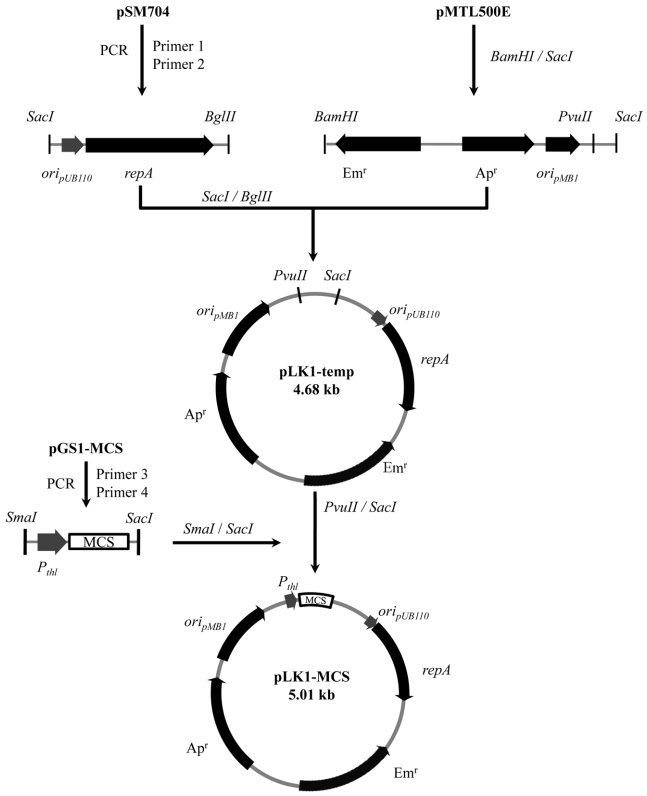

Fig. 1. Construction scheme for the shuttle plasmid pLK1-MCS.

The ori_{pUB110} and the *repA* gene encoding the replication initiator originated from plasmid pSM704, and the ampicillin resistance (Ap^r) and erythromycin resistance (Em^r) genes, together with ori_{pMB1}, originated from plasmid pMTL500E. P_{Thl} denotes the promoter region of the thiolase gene in *C. acetobutylicum* ATCC 824.

Electroporation

Electroporation was performed as previously described [19]. *C. acetobutylicum* was cultured anaerobically to an OD_{600} of 1.0 in CGM (pH 5.8) at 37°C. The cells were collected by centrifugation at 3,000 ×*g* (5702R; Effendorf, Hamburg, Germany) for 20 min at 4°C after storing on ice for 10 min. The cell pellet was washed three times with electroporation buffer (270 mM sucrose and 686 mM NaH_2PO_4, pH 7.4, ice-chilled before use) and then resuspended in 2 ml of the buffer. All plasmid DNAs were methylated in *E. coli* TOP10 (pAN1) before transformation into *C. acetobutylicum* for protection from the *C. acetobutylicum* restriction system [18]. Electroporation was performed using a Gene Pulser II (Bio-Rad, Hercules, CA, USA; 2.5 kV, ∞ Ω, and 25 μF) with 4 mm electrode gap cuvettes (Bio-Rad) containing electrocompetent cells and plasmid DNA (5 μg). After the electroporation, 1 ml of 2× YTG medium was added to the mixture and incubated anaerobically at 37°C for 4 h. The cells were then spread onto CGM agar containing 40 μg/ml erythromycin and incubated in an anaerobic chamber at 37°C.

Plasmid Stability

The segregational stability of pLK1-MCS, pGS1-MCS, and pMTL500E was determined with slight modification of a previous protocol [28]. A single colony of *C. acetobutylicum* ATCC 824 harboring each plasmid was inoculated into a 50 ml conical tube containing 40 ml of liquid CGM without antibiotics and cultured anaerobically at 37°C for 24 h. The cells were diluted and spread onto CGM agar plates without antibiotics so that the cell numbers could be counted. After 36 h of incubation, 50 colonies were transferred from the CGM agar plate to a CGM agar plate containing erythromycin (40 μg/ml) by replica plating to count the colonies and to determine segregational stability. Relative segregational stability was determined as follows:

$$S_r(\%) = \frac{N_e}{N_t} \times 100$$

where S_r, N_t, and N_e represent relative segregational stability, total colony number replica plated, and the colony number grown on

replica plate containing erythromycin after 36 h incubation, respectively.

Forty microliters of the resting cell broth was inoculated into 40 ml of fresh liquid CGM in order to achieve a 1:1,000 dilution. Since the cells were diluted 1,000 times at the end of each cycle, it was assumed that one cycle represented approximately 10 generations. After 100 generations, the shuttle plasmids were isolated from *C. acetobutylicum* and subsequently re-transformed into competent *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) cells. All plasmid DNA isolations were performed using the Wizard Plus SV miniprep kit (Promega). Plasmid DNAs isolated from *E. coli* XL1-Blue were fully sequenced by primer walking (Solgent, Daejeon, Korea).

Fermentation

Batch fermentation was carried out in a Bioflo 310 bioreactor (Effendorf, Hamburg, Germany) containing 1.6 L of CGM supplemented with 80 g/l glucose. A single colony was grown anaerobically to an OD_{600} of 1.0. This seed culture was transferred to a 500 ml flask containing 360 ml of CGM. When the cell density reached an OD_{600} of 1.0, the flask culture was inoculated into the bioreactor. The pH was kept above 5.0 and controlled with an ammonia solution without dilution during fermentation. The temperature and agitation speed were controlled at 37°C and 200 rpm, respectively. During fermentation, the bioreactor was flushed with oxygen-free nitrogen at a flow rate of 50 ml/min to maintain anaerobic conditions.

Analysis of Metabolites

Cell growth was monitored by measuring the OD_{600} using a DR5000 spectrophotometer (HACH, Loveland, CO, USA). Metabolites were determined using a gas chromatography system (Agilent 6890N, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a packed column (80/120 Carbopack BAW glass column; Supelco, St. Louis, MO, USA). Helium gas was used as a carrier phase at a flow rate of 20 ml/min with the septum purge flow of 3 ml/min. Conditions for metabolite analysis were as follows: inlet heater temperature at 250°C; oven temperature of 90°C for 2 min, ramping to 170°C at 5°C/min, and holding at 170°C for 2 min; and FID at 280°C. The glucose concentration was analyzed using a high-performance liquid chromatography system (Agilent 1200 series) equipped with a reflective index detector and an Aminex-87H column (Bio-Rad). A 0.01 N H_2SO_4 solution was used as the mobile phase at a flow rate of 0.6 ml/min and the oven temperature was adjusted to 80°C for optimal column performance.

Results and Discussion

Construction of *C. acetobutylicum-E. coli* Shuttle Plasmid

Many replicons originating from various gram-positive bacteria can replicate in Clostridia [7, 12, 25, 27, 29, 32]; however, only a few replicons (*i.e.*, pAMβ1, pIM13, and pT127) can be maintained in *C. acetobutylicum* [5]. Among them, pIM13 and pT127 are listed as class I staphylococcal plasmids. The class I plasmids can replicate in many gram-positive bacteria, including staphylococci, bacilli, and streptococci. For example, pIM13 can replicate in *Staphylococcus aureus*, *Bacillus subtilis*, and *C. acetobutylicum* [18, 22]. The cryptic plasmid pUB110, which was originally found in *S. aureus* [2], can replicate in *B. subtilis* [8, 11] and in *C. beijerinckii* SA-1 (ATCC 35702, formerly known as *C. acetobutylicum* SA-1) [17]. Therefore, we examined the properties of pUB110 as a replicon for *C. acetobutylicum*. The pLK1-MCS shuttle plasmid was constructed by combining a partial pUB110 fragment, which included the replication origin (ori_{pUB110}) and replication initiator gene (*repA*) from plasmid pSM704, with the *E. coli* pMB1 origin (ori_{pMB1}) and the selection markers (*i.e.*, erythromycin and ampicillin resistance genes; Em^r and Ap^r) from the shuttle plasmid pMTL500E (Fig. 1). In addition, the shuttle plasmid contained the promoter (P_{Thl}) of the *thiolase* gene from *C. acetobutylicum* ATCC 824 and the RBS to allow expression of a recombinant gene. pLK1-MCS was transferred into Clostridia for further study.

Transfer Frequency and Host Spectrum of the Shuttle Plasmid

Each of the plasmids pLK1-MCS, pMTL500E, and pGS1-MCS was transformed individually into *C. acetobutylicum* ATCC 824, *C. beijerinckii* NCIMB 8025, and *C. saccharobutylicum* NCP 262, and the transformation efficiencies were calculated (Table 3). The transfer frequency in *C. acetobutylicum* was similar among the plasmids. However, pLK1-MCS could not replicate in either *C. beijerinckii* or *C. saccharobutylicum*. A previous report suggested that pUB110 can replicate in *C. beijerinckii* SA-1 [17], which is not consistent with our results. This inconsistency may be due to host differences between SA-1 and NCIMB 8052, or may be because pLK1-MCS did not contain the appropriate DNA region necessary for replication in *C. beijerinckii* NCIMB 8052. In fact, pUB110 has two replication origins; a minus origin

Table 3. Transfer frequency of shuttle plasmids into Clostridia.

Plasmid	[a]Clostridial strains		
	ATCC 824	NCIMB 8052	NCP 262
pLK1-MCS	4.3×10^2	-	-
pMTL500E	2.6×10^2	3.6×10^3	-
pGS1-MCS	4.6×10^2	-	-

[a]The values are transformant colonies per 10 μg plasmid DNA.

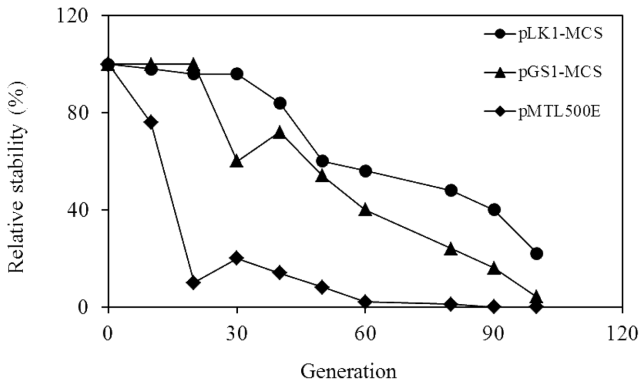

Fig. 2. Comparison of the segregational stability of three shuttle plasmids, pLK1-MCS, pGS1-MCS, and pMTL500E, in *C. acetobutylicum* ATCC 824 for 100 generations.

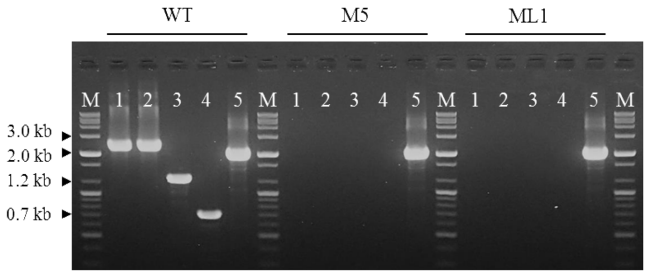

Fig. 3. Confirmation of loss of pSOL1 in *C. acetobutylicum* ML1. Results from PCR using ATCC 824 (WT, wild type) indicate that pSOL1 is present in these cells. Results using ML1 and M5 indicate that pSOL1 is absent from these cells. The *ptbbu*k operon, which resides on the chromosome of *C. acetobutylicum*, was amplified from DNA templates to confirm that the prepared DNA templates were derived from *C. acetobutylicum* strains. Lane M, 1 kb ladder; 1, *adhE1* (2.6 kb); 2, *adhE2* (2.6 kb); 3, *ctfAB* (1.3 kb); 4, *adc* (0.7 kb); 5, *ptbbuk* (2.0 kb).

and a plus origin [1]. In this study, we used the plus origin to construct the pLK1-MCS shuttle plasmid. Recently, the restriction mechanism of *C. saccharobutylicum* was described and found to differ from that of *C. acetobutylicum* [16]. The inability to transform *C. saccharobutylicum* with the shuttle plasmid pLK1-MCS may be due to improper methylation by phi-3T methyltransferase (*Mtase*). As a result, pLK1-MCS replicated only in *C. acetobutylicum* ATCC 824.

Segregational Stability of Plasmid pLK1-MCS

The transformants carrying pLK1-MCS (pUB110 replicon), pGS1-MCS (pIM13 replicon), and pMTL500E (pAMβ1 replicon) were used to examine the segregational stability of the plasmids during liquid culture in the absence of antibiotics for 100 generations. Cells were plated on CGM agar plates without antibiotics, and 50 colonies were selected to identify plasmid loss by replica plating onto plates containing erythromycin for every 10 generations (Fig. 2). pLK1-MCS showed superior segregational stability compared with pGS1-MCS and pMTL500E. After the segregational test of 100 generations, pLK1-MCS was isolated from *C. acetobutylicum* and re-transformed to *E. coli* XL1-Blue to analyze the sequence of the re-transformed plasmid. It was found that pLK1-MCS was structurally stable in *C. acetobutylicum* without any mutation, including deletion, rearrangement, or point mutation during the 100 generations.

Complementation of *adhE1-ctfAB-adc* Operon in *C. acetobutylicum* ML1

Recently, we isolated mutant *C. acetobutylicum* ATCC 824 (designated as *C. acetobutylicum* ML1). This mutant was found to lack the pSOL1 megaplasmid, which contains four genes (*i.e.*, *adhE1*, *adhE2*, *ctfAB*, and *adc*) required for ABE production. The loss of pSOL1 in *C. acetobutylicum* ML1 was confirmed by PCR with the appropriate primer pairs (Table 2). The megaplasmid loss in *C. acetobutylicum* ML1 was further confirmed by PCR with primers 15 and 16, which bind to the oxidoreductase gene in pSOL1 (Fig. 3 and Table 2). *C. acetobutylicum* ML1 does not produce ABE, owing to the loss of megaplasmid pSOL1 (Table 4), as was previously described for *C. acetobutylicum* M5 [3]. The previous studies reported that M5 could be complemented for ABE production by homologous expression of *adhE1*, *ctfAB*, and *adc* genes [4, 23]. To prove the practical stability of our shuttle plasmid in *C. acetobutylicum*, pLK1-E1ABC was constructed and introduced into *C. acetobutylicum* ML1. In order to identify genetic complementation and to

Table 4. Concentration of metabolites produced by recombinant *C. acetobutylicum* in batch fermentation.

Strain	Cell density (OD_{600})	Metabolite concentration (g/l)				
		Acetone	Ethanol	Butanol	Acetate	Butyrate
ATCC 824	10.16	3.19	0.74	10.17	5.31	3.88
ML1 (pLK1-MCS)	10.58	0.04	0.36	0.11	6.47	11.84
ML1 (pLK1-E1ABC)	10.20	1.21	3.06	6.21	5.69	7.85

confirm ABE production, batch fermentations were performed for 42 h. Although the titers of solvent produced by transformed ML1 were not similar to the titers obtained from wild types, substantial levels of all solvents (*i.e.*, acetone, butanol, and ethanol) were measured (Table 4). After fermentation, the recombinant plasmids were isolated and re-transformed into *E. coli* XL1-Blue for sequence analysis. The results showed that the plasmid was stably maintained in ML1 without recombination or mutation. From the segregational stability and complementation experiments, we confirmed that the shuttle plasmid can stably replicate in *C. acetobutylicum*.

In conclusion, a new *C. acetobutylicum-E. coli* shuttle plasmid, pLK1-MCS, was constructed by combining the pUB110 and pMB1 replicons. The shuttle plasmid is more stably maintained than pMTL500E (pAMβ1 replicon) and pGS1-MCS (pIM13 replicon) and is structurally stable in *C. acetobutylicum* for 100 generations without mutation. Recombinant genes in the shuttle plasmid can be functionally expressed in *C. acetobutylicum* ML1. Therefore, the shuttle plasmid is useful as a genetic tool in industrial biotechnology using *C. acetobutylicum* and *E. coli*.

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