Characterization of a cryptic plasmid isolated from *Lactobacillus casei* CP002616 and construction of shuttle vectors based on its replicon

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ABSTRACT

The cryptic plasmid pLC2W was isolated from *Lactobacillus casei* CP002616. Nucleotide sequence analysis revealed that 4 putative open reading frames (ORF) were responsible for DNA replication. Four *Escherichia coli*-*Lactobacillus* shuttle vectors were constructed using different lengths of the pLC2W replicon to identify the shortest functional replicon. The length of the pLC2W replicon did not affect the stability of the plasmids. Green fluorescent protein (GFP) as a reporter was expressed successfully in several lactobacilli using our constructed vectors. The results suggested that the expression vectors pUE-F0GFP and pUE-F1GFP are potential molecular tools for heterologous gene cloning and expression in lactobacilli. Moreover, 2 plasmid-curing methods were used to eliminate pLC2W from *L. casei*. We detected no difference between *L. casei* CP002616 and *L. casei* CP002616 pLC2WΔ-IC (mutant strain cured by plasmid incompatibility method) in production of exopolysaccharide (EPS) or acid. However, EPS and acid production were both reduced in *L. casei* CP002616 pLC2WΔ-HT (mutant strain cured by high-temperature heat treatment method), demonstrating a difference between these 2 curing methods. Sequence analysis of pLC2W and plasmid curing data suggest that plasmid pLC2W is not involved in EPS synthesis.

Key words: cryptic plasmid pLC2W, shuttle vector, *Lactobacillus casei*, expression vector, cure plasmid

INTRODUCTION

Lactic acid bacteria (LAB) are widespread in natural environments. They play important roles in human

health and the human gastrointestinal tract with a long history (de Vos, 2011; Bourdichon et al., 2012; Gaspar et al., 2013). Lactic acid bacteria can improve lactose digestion, enhance cellular immunity, help to cure disease in humans, and protect against colon cancer (Wen et al., 2012; Chen et al., 2014; Aktas et al., 2016; Gleeson et al., 2016; Kato-Kataoka et al., 2016). Although LAB are useful in many applications, the mechanism by which lactobacilli contribute to other bacteria and hosts remains obscure (Vaughan et al., 2000). Many researchers have focused on the genetic mechanisms in LAB, and a wide range of genetic tools have been developed in LAB and successfully applied in several LAB (Mathiesen et al., 2004).

Because genetic elements in LAB are related to plasmids, shuttle plasmids have been constructed and applied to express important genes. Plasmids isolated from LAB are the best choice as the backbone of shuttle vectors. Recently, many cryptic plasmids have been isolated from LAB, including pCD01 and pCD02 from *Lactobacillus paracasei* (Desmond et al., 2005), pLC494 from *Lactobacillus casei* (An and Miyamoto, 2006), pRV500 from *Lactobacillus sakei* (Crutz-Le Coq and Zagorec, 2008), pDOJ1 from *Lactobacillus delbrueckii* (Lee et al., 2007), pM4 from *Lactobacillus plantarum* (Yin et al., 2008), and pBC1 from *Bifidobacterium catenulatum* (Alvarez-Martín et al., 2007). Many shuttle vectors have been constructed based on these cryptic plasmids. Most *Lactobacillus* plasmids duplicate via a theta or rolling circle (RC) mechanism, including the plasmids mentioned above. Theta-type plasmids can accommodate insertions of larger DNA fragments than RC-type plasmids.

Some reports have noted that plasmids in lactobacilli are involved in production of exopolysaccharides (EPS; Vedamuthu and Neville, 1986). To study the relationship between pLC2W and EPS production, we used 2 plasmid-curing methods for pLC2W. The current common approach for curing plasmid is a high-

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temperature method, which is cumbersome and time consuming (Yin et al., 2008; Sun et al., 2010; Chen et al., 2014). Mutations may occur during the harsh physical curing process. For these reasons, we also used a new rapid and high-efficiency curing method based on plasmid incompatibility—the inability of 2 plasmids with the same replicon to coexist stably in the same host (Liu et al., 2012). Introducing a smaller plasmid with the same replicon will cure the original plasmid, especially if antibiotics are also present. This method has been successfully used in many strains to generate plasmid-free mutants (Uraji et al., 2002; Ni et al., 2008; Liu et al., 2012) and is a powerful and robust method of plasmid curing.

We recently isolated a new *L. casei* strain from traditional dairy products in Inner Mongolia designated *L. casei* CP002616. The aims of the current study were (1) to predict the replication mechanism of pLC2W (a cryptic plasmid isolated from *L. casei* CP002616) using phylogenetic analysis of the replication protein; and (2) to confirm the shortest functional replicon by constructing a series of plasmids based on different lengths of the pLC2W putative replicon. We determined the host range of the new shuttle vector and constructed a new expression vector based on it. We also used 2 methods to cure plasmid of *L. casei* CP002616 and compared the production of EPS and acid in *L. casei* CP002616 and mutants.

MATERIALS AND METHODS

Bacterial Strains, Enzymes, and Kits

All plasmids were introduced into competent *Escherichia coli* cells (*E. coli* Top10, Takara, Tokyo, Japan) grown in Luria-Bertani broth (**LB**) medium at 37°C. The strains and plasmids used in this study are shown in Table 1. *Lactobacillus* strains were grown in de Man, Rogosa, and Sharpe (**MRS**) medium (1 L of the medium contained 10.0 g of peptone, 10.0 g of beef extract, 4.0 g of yeast extract, 20.0 g of glucose, 1 mL of Tween-80, 2.0 g of dipotassium hydrogen phosphate, 5.0 g of sodium acetate, 2.0 g of triammonium citrate, 0.2 g of magnesium sulfate, and 0.05 g of manganese sulfate) at 37°C (*Lactococcus lactis* was grown at 30°C) and pH 6.2 without shaking. Antibiotics were used, when necessary, as follows: ampicillin (**Amp**; Sangon Biotech, Shanghai, China) at 100 µg/mL for *E. coli*; erythromycin (**Em**; Sangon Biotech) at 200 µg/mL for *E. coli* and 10 µg/mL for *Lactobacillus*.

The PCR used KOD-plus-neo DNA polymerase (Toyobo, Osaka, Japan) or KOD FX polymerase (Toyobo). A ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co. Ltd., Nanjing, China) and DNA restriction enzymes (Thermo Fisher Scientific, Waltham, MA) were used to prepare clones. Plasmids and chromosomal DNA of *E. coli* were extracted using

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description ¹	Reference
Strain		
<i>Lactobacillus casei</i> CP002616	Wild-type strain	CP002616, Inner Mongolia, China
<i>Lactobacillus casei</i> BDII	Transformation host	CP002618, Inner Mongolia, China
<i>Lactobacillus casei</i> AR341	Transformation host	Our laboratory
<i>Lactobacillus paracasei</i> AR93	Transformation host	Our laboratory
<i>Lactococcus lactis</i> NZ9000	Transformation host	CP002094
<i>Lactobacillus plantarum</i> (WCFS1)	Transformation host	Kleerebezem et al. (2003)
<i>Lactobacillus brevis</i>	Transformation host	CGMCC1.2028
<i>E. coli</i> Top10	Transformation host	Takara (Tokyo, Japan)
Plasmid		
pUC19	Amp ^r , ori	Takara (Tokyo, Japan)
pMG36e	Em ^r	van de Guchte et al. (1989)
pEGFP-N1	For eGFP gene cloning	Cormack et al. (1996)
pLC2W	Plasmid of <i>Lb. casei</i> CP002616	Chen et al. (2011)
pUE-F0	Clone total replicon, em ^r into pUC19	This work
pUE-F1	Ori and repA, em ^r cloned into pUC19	This work
pNZ5319	Cm ^r	Lambert et al. (2007)
pUCM-F1	Cm ^r replaced em ^r of pUE-F1	This work
pUE-F2	Ori, orf1, orf2 and orf3, em ^r cloned into pUC19	This work
pUE-F3	Ori, orf1 and orf2, em ^r cloned into pUC19	This work
pUE-F4	Ori and em ^r cloned into pUC19	This work
pUEG-F1-P23	Promoter P23 and eGFP cloned into pUE-F1	This work
pUEG-F1-Pldh	Promoter Pldh and eGFP cloned into pUE-F1	This work
pIB184-GFP	eGFP cloned into pIB184	This work

¹Amp^r = ampicillin resistance; ori = origin; Em^r = erythromycin resistance; eGFP = enhanced green fluorescent protein; rep = replicon; Cm^r = chloramphenicol resistance; orf = open reading frame.

kits (Axygen Biotechnology Co. Ltd., Hangzhou, China). Plasmids of *Lactobacillus* were extracted according to the method described by O'Sullivan and Klaenhammer (1993), and genomic DNA of *Lactobacillus* was extracted by a method described previously (Chen et al., 2014). All enzymes were used following the instructions of the manufacturers.

Sequence Analysis of Replication Initiation Protein

To assess the replication mechanism of pLC2W, we compared the AA sequence of the replication initiation (**Rep**) protein of pLC2W with those of Rep proteins in the theta-type plasmids and RC plasmids in *Lactobacillus*. The Rep sequences were first aligned using Clustal X software (version 2.0, Conway Institute University College Dublin, Ireland; Larkin et al., 2007), and a phylogenetic tree was constructed by the neighbor-joining method using MEGA software (version 6.0, Biodesign Institute, Arizona State University, Tempe, AZ). The bootstrap consisted of 1,000 replications to assess the reliability level of the nodes of the tree (Tamura et al., 2007).

Electroporation Transformation

Competent cells of *L. casei* and *L. paracasei* were prepared as follows: 2.5 mL of overnight culture was inoculated in 50 mL of MRS with 1% glycine (MRSG) and incubated at 37°C without shaking until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8. Cells were harvested by centrifugation at $5,000 \times g$ for 10 min. Cells were washed twice in an ice-cold washing solution containing 10% glycerol, with centrifugation at $5,500 \times g$ for 10 min. The cells were then resuspended in 0.5 mL of the same solution for electroporation. For each transformation, 50 μ L of cell suspension was mixed with ~100 ng of plasmid DNA and incubated on ice for 10 min. The mixture was then transferred to a 2-mm cuvette (Bio-Rad Laboratories, Hercules, CA), and electroporation was performed at 2 kV, 200 k Ω , and 25 μ F, using an Eppendorf Multiporator (Eppendorf, Hamburg, Germany). Then, 950 μ L of warmed MMRS broth (MRS + 500 mmol/L saccharose + 20 mmol/L $MgCl_2$ + 2 mmol/L $CaCl_2$) was added to the cuvette, and the transformed cells were allowed to recover by incubation at 37°C for 2 to 3 h. The cells were then plated on MRS agar plates with erythromycin to recover transformants. Transformation rates were expressed as the number of transformants per microgram of plasmid DNA. Competent cells of *L. plantarum* WCFS1 and *L. lactis* NZ9000 were prepared as the method described previously (Serror et al., 2002; Papagianni et al., 2007; Luxananil et al., 2009; Yin et al., 2009).

Determination of Plasmid Copy Number

Relative copy numbers of pLC2W and its derived plasmids in *L. casei* CP002616 and other *L. casei* strains were estimated using real-time quantitative PCR (qPCR) using SYBR Green (Bio-Rad Laboratories) and MyIQ2 (Bio-Rad). The elongation factor gene *DnaA* was used as a chromosomal reference, and *repA* of pLC2W was used as the target gene. Primers for qPCR were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA) to give PCR products of similar sizes. A 126-bp product was designed for *repA* and an 84-bp product was designed for *DnaA*, with the primers listed in Table 2. The copy number of pLC2W was calculated using the formula: plasmid copy number (PCN) = $E^{(C_{tc} - C_{tp})}$ (Skulj et al., 2008), where E represents the PCR amplification efficiency of the target, p and c indicate plasmid and chromosome respectively, and Ct indicates = cycle threshold value; E could be calculated using the slope of the standard curve and the equation $E = 10^{-1/\text{slope}}$ (E was 2 when the product amplified at 100% efficiency; Pfaffl, 2001).

Construction of *E. coli*-*Lactobacillus* Shuttle Vectors

The oligonucleotide primers used for the construction of the cloning vector are listed in Table 2. Plasmid pUC19 was double-digested with *SacI* and *BamHI*. The Em resistance gene was amplified by PCR using the primers EmF and EmR, and pMG36e as the template. Then, DNA fragments of different lengths were produced by PCR amplification using pLC2W DNA as the template. All DNA fragments were ligated by T4 Ligase (Sangon Biotech). Plasmids were constructed using different lengths of the pLC2W replicon.

The Em resistance gene of pUE-F1 was replaced in a 2-step process by a chloramphenicol (**Cm**) resistance gene from pNZ5319, giving rise to pUCM-F1. The availability of constructs with different antibiotic selections allowed the consecutive transformation of pUE-F1 and pUCM-F1 into LAB.

Host Range and Segregation Stability

The concentrations of plasmid DNA used for transformation were quantified using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). One hundred nanograms of plasmid was used to transform into *L. casei* CP002616, *L. casei* AR341, *L. casei* BD II, *L. paracasei* AR93, *L. plantarum* WCFS1, *L. brevis* CGMCC1.2028, and *Lc. lactis* NZ9000 by electroporation. The transformation efficiency was measured by calculating the transformants per microgram of DNA (Sun et al., 2010).

To determine the segregation stability of the vectors, plasmid-bearing strains were grown overnight in MRS with Em, and 1% of the overnight culture was inoculated into MRS without antibiotic. After incubation for about 20, 40, 60 or 80 generations, counts (cfu) were determined by using nonselective and selective MRS agar plates. Ratios of counts on the selective to nonselective plates were used to estimate the stability of different plasmids as described previously (Alvarez-Martín et al., 2007; Sun et al., 2010; Chen et al., 2014).

Construction of Expression Vector

To construct a new expression vector with pUE-F1, 3 additional DNA elements were required; namely, a promoter element and 2 multiple cloning sites. The P₂₃ promoter was amplified by using primers P23-up and P23-down, and plasmid pIB184 was used as the template. To verify that the vector was an effective reporter system in *L. casei*, the lactic dehydrogenase (*ldh*) promoter was amplified with primers pldh-up and pldh-down, and *L. casei* CP002616 genomic DNA was used as a template. An enhanced green fluorescent protein (eGFP) gene was amplified from plasmid pEGFP-N1 (Cormack et al., 1996) with primers GFP-up and GFP-down. All primer pairs are listed in Table 2. The 4 amplicons were then cloned into pUE-F1 with double digests at *SpeI* and *BamHI* to obtain 2 new plasmids: pUEG-F1-P₂₃ and pUEG-F1-P_{ldh}.

The efficiencies of the different promoters in *L. casei* CP002616 were estimated using qPCR. The elongation factor gene *DnaA* (GenBank accession no. 501467306) was used as a chromosomal reference, and *GFP* was used as the target gene.

Plasmid Curing from *L. casei* CP002616

Two different methods of plasmid curing were used to eliminate pLC2W. For the HT method, *L. casei* CP002616 was propagated in MRS at 42°C for about 200 generations. Then, the culture was diluted and plated on MRS plates. To identify plasmid-free clones, colony PCR was conducted using primers (repA-up/down) that can amplify the fragment only present in the plasmid. For the IC method, the incompatible plasmid pUE-F1 was transferred into competent cells of *L. casei* CP002616 by electroporation. Cells were grown on MRS plates with Em for 48 h. One positive transformant along with pUE-F1 grew in MRS with Em for at least 5 culture passages. Antibiotic concentration increased 1.5 times when cells were transferred each time. The culture was diluted and plated on MRS-Em plates, and positive transformants were selected using primers (rmp-up/down) that can amplify the pLC2W specific fragment.

To obtain a plasmid-cured strain without exogenous DNA, we needed to cure pUE-F1 from pLC2W-cured *L. casei* CP002616. The host was propagated twice in 4

Table 2. Oligonucleotides and primers¹

Name	Oligonucleotide (5'–3')
Em-F	TAGGCGTATCACGAGGCCCTTTTCGTCGGATCCAGGTACTAAAACAATTCATCC (<i>BamHI</i>)
Em-R	TCTTCATTTGCGCACCGAAACCGGAGGTACCGCTACCAAGACGAAGAGGATG (<i>KpnI</i>)
GFP-up	TATAATGAAAAATATGAATGACAATGATGTTCCATGGTGAGCAAGGGCGA
GFP-down	ATATCAGATCTTACTTGTAACAGCTCGTC
p23-up	TTCCATATAATCCTATAAAATCCCGGGCGCGCTCGAGGGCCCCGAAAAGCCCTG
p23-down	AACAGCTCCTCGCCCTTGCTCACCATGGAACATCATTGTCATTCATA
p23-GFP-up	ATTTTACTGGATGAATTGTTTTAGTACCTGGATCCTGTAAAACGACGGCCAGTCTGT
p23-GFP-down	AACAGCTATGACCATGATTACGCCAAGCTTGCATGCAGGAAACAGCTATGACCGCT
pldh-up	ATAAAATCCCGGGCGGCCGCTCGAGGGCCCACGGATTTTGCCTTTTCC (<i>ApaI</i>)
pldh-down	TGAACAGCTCCTCGCCCTTGCTCACCATGGGGTGATATCATCCTTTCT (<i>NcoI</i>)
repC2-F	ATCCTCTTCATCTCTTCGTCTTGGTAGCGGTACCTCCGGTTTCGGTGCGCA (<i>KpnI</i>)
repC2-F1	ATCGACGGGGTACCAGCCATATAAATACCGCCTTCC (<i>KpnI</i>)
repC2-F2	ACTGCGGGGTACCACATAATCGTCTGTAACTTTT (<i>KpnI</i>)
repC2-F3	ATTGCGGGGTACCGATGGTCAATAATCGGTTG (<i>KpnI</i>)
repC2-F4	ATCGACGGGGTACCCTAAAGAGTTTAATCAACGG (<i>KpnI</i>)
repC2-R	TCTCCCCGCGCGTTGGCCGATTTCATTAATGGAGCTCTTGCTGTGCAGAAATCAAATGG (<i>SacI</i>)
RepA-F	GCTTCATTCTCGCGTTTGTA
RepA-R	AGCAAACCTCCTCTTCCAGA
DnaA-F	TAAGGGCTTGCTGTGCGATG
DnaA-R	AGATCTTCAGCATCGGCTTT
rmp-up	ATGTCAGAAAAAACGC
rmp-down	CATAGTCCCGTGTTCCCTGC
repA-up	TTAGAATTCATCAGGGTCGCC
repA-down	GAACCCAATAAATTCGAGT

¹Underlining indicates digestion sites.

mL of MRS without antibiotics. Cultures were diluted and spread on MRS plates without antibiotics for 48 h. Clones were streaked onto MRS plates with or without erythromycin and incubated at 37°C for 36 h.

EPS and Acid Production

To analyze the EPS production of the mutant strains, fermentation was carried out with *L. casei* CP002616 pLC2WΔ-HT, *L. casei* CP002616 pLC2WΔ-IC, and *L. casei* CP002616 strains in 30 mL of liquid MRS medium for 24 h. The cells were removed by centrifugation at $10,000 \times g$ for 20 min (4°C), and EPS was precipitated from the supernatant with cold 95% ethanol for 24 h at 4°C. The EPS fraction was resuspended in ultrapure water (10%, wt/wt) after centrifugation ($10,000 \times g$, 20 min, 4°C), and EPS was dialyzed against water for 72 h using a membrane with molecular weight cut-off of 12,000 to 14,000 Da. The production of EPS was evaluated as previously described (Lokman et al., 1997). The different types of EPS from *L. casei* CP002616 pLC2WΔ-HT, *L. casei* CP002616 pLC2WΔ-IC, and *L. casei* CP002616 were named EPS-HT, EPS-IC, and EPS-WT, respectively.

High-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD; Dionex ICS-5000 System, Dionex Corporation, Sunnyvale, CA) was used to identify and quantify monosaccharides of EPS-HT, EPS-IC, and EPS-WT. The EPS were dissolved in 0.5 mL of 12 M H₂SO₄ at an ice bath for 30 min, and then diluted to 3 mL (2 M H₂SO₄) to further hydrolyze for 2 h at 100°C. Separation was performed on a CarboPac PA20 column (3 mm × 150 mm, Dionex Corp.) and a CarboPac PA20 Guard (3 mm 30 mm, Dionex Corp.) with a gradient elution procedure at a flow rate of 0.5 mL/min at 30°C. The eluents consisted of 250 mM NaOH solution (A), distilled water (B), and 1 M sodium acetate (C). Initially, 0.8% A was eluted for 20 min, followed by a gradient increase from 5% C to 20% C while maintaining 0.8% A. Finally, 80% A was eluted to regenerate the column for 20 min (Yuan et al., 2015). Chromeleon software (Dionex Corp.) was used to process the data.

The molecular weight distributions of EPS-HT, EPS-IC, and EPS-WT were determined by high-performance gel-permeation chromatography using a Waters instrument (Waters Corp., Milford, MA) equipped with a refractive index detector (Waters 2414), a variable wavelength detector (Waters 2489), and an Ultrahydrogel Linear column (7.8 × 300 mm; Waters Corp.). The polysaccharides were eluted with 0.1 M sodium nitrate at a flow rate of 0.6 mL/min. The column temperature was maintained at $35.0 \pm 0.5^\circ\text{C}$. The polysaccharides

were filtered through a 0.45-μm membrane before injection. The molecular weight of the samples was calculated according to the calibration curve established using dextran standards (2×10^6 , 300,600, 135,350, 64,650, 36,800, 13,050, and 9,750).

To determine the acid production of the mutants, freshly grown overnight cultures of *L. casei* CP002616 pLC2WΔ-HT, *L. casei* CP002616 pLC2WΔ-IC, and *L. casei* CP002616 were inoculated into MRS at 2% and incubated at 37°C. The change in pH was monitored using a pH meter every 2 h.

RESULTS

Molecular Characterization of pLC2W

Plasmid pLC2W is a native plasmid of *L. casei* CP002616 isolated from traditional dairy products in Inner Mongolia. The plasmid was 38,392 bp with a GC content of 42.64% (Chen et al., 2011); it was predicted to have 4 putative open reading frames (ORF). Analysis of the AA sequences of these ORF showed that they shared 100% identity with the replication proteins on pBD II and pW56 (Ai et al., 2011; Hochwind et al., 2012): ORF1 (351 AA) was identified as a replication protein (RepA), ORF2 (278 AA) was identified as a replication-associated protein (RepB), and ORF3 (129 AA) and ORF4 (113 AA) were identified as hypothetical proteins with unknown functions.

Strikingly, RepA and RepB had very low sequence similarity to each other, exhibiting 44% identity and 10% coverage using National Center for Biotechnology Information blastp (Protein BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A phylogenetic tree was constructed using the AA sequences of RepA and the other 11 Rep proteins from the *Lactobacillus* plasmids that replicate via theta or RC replication (Figure 1). Based on phylogenetic analysis, RepA of pLC2W was from the RC replication group; therefore, we reasoned that the replicon of pLC2W could replicate via RC replication.

Shortest Functional Replicon and Copy Number of pLC2W and Derivatives

To investigate the shortest functional replicon of pLC2W, we constructed a series of shuttle plasmids (pUE-F0, pUE-F1, pUE-F2, pUE-F3, and pUE-F4) with different sizes of pLC2W replicon (Figure 2a). These plasmids were checked on LB plates containing Amp. Subsequently, the recombined plasmids were transformed into *L. casei* CP002616 to study the origin activity of the different fragments. The stability of these shuttle plasmids was then estimated by scoring

for plasmid-free colonies after continuous incubation in MRS for several generations under nonselective conditions. The results are summarized in Table 3.

Four shuttle plasmids (all except pUE-F4) with different lengths of replicon in *L. casei* CP002616 formed Em-resistant colonies. The transformation efficiency of these plasmids was about 10^5 cfu/ μ g of DNA, and we detected no significant difference in stability between the shuttle plasmids. In summary, the minimal replicon comprised the origin (Ori) and RepA, and replication activity required both elements. Plasmid pUCM-F1 was obtained for the consecutive transformation of pUE-F1 and pUCM-F1 into LAB (Figure 2b).

The qPCR was used to determine the relative copy numbers of pLC2W and its derived shuttle plasmids in *Lactobacillus* cells. Two sets of primers were designed to generate PCR products for the *repA* and *DnaA* genes, which were used to quantify plasmids and chromosomes in the samples, respectively. Analysis of the results revealed that the copy number of pLC2W was 4.82 copies per cell. The estimated copy number of the derived plasmid pUE-F1 in *L. casei* CP002616 was 33.9 copies per cell.

Host Range and Segregation Stability

Because we detected no significant difference between the constructed plasmids, we selected pUE-F1 for transforming into different *Lactobacillus* strains to study the host range. The tested strains included *L. casei* CP002616, *L. plantarum* WCFS1, *L. brevis* (CGMCC 1.2028), *L. casei* BDII, *L. casei* AR341, *L. paracasei* AR93 and *Lactococcus lactis* NZ9000. Plasmid pUE-F1 survived in *L. casei* CP002616, *L. casei* BDII, *L. casei* AR341, *Lc. lactis* NZ9000, and *L. paracasei* AR93, but did not survive in *L. plantarum* WCFS1

or *L. brevis* (CGMCC 1.2028). The efficiencies ranged between 10^2 and 2.2×10^5 cfu/ μ g DNA (Table 4). We then assessed stability of the pLC2W-derived shuttle plasmids in different hosts; pUE-F1 was unstable in *L. casei* CP002616, *L. casei* BDII, and *L. casei* AR341 but stable in *L. paracasei* AR93. The stability of pUE-F1 in *Lc. lactis* NZ9000 was not estimated in this work.

Expression of the GFP Gene in a *Lactobacillus* Host

Two strong promoters (P_{23} and P_{ldh}) were used to construct new expression vectors, named pUEG-F1- P_{23} and pUEG-F1- P_{ldh} (Figure 2c). These plasmids were transformed into competent cells of *L. casei* CP002616 and *Lc. lactis* NZ9000 (Figure 3a and b). Green fluorescence was observed in several recombinant cells. To further determine GFP expression of different promoters, we used qPCR for quantitative analysis. Expression of GFP in pUEG-F1- P_{ldh} was defined as 1, and the expression of pIB184-GFP and pUEG-F1- P_{23} was 1.804 and 1.027, respectively (Figure 3c). We detected no significant difference in GFP expression between promoters P_{23} and P_{ldh} .

Plasmid Curing from *L. casei* CP002616

To study the relationship between plasmid and EPS production in *L. casei* CP002616, we cured pLC2W using 2 different methods and compared them. For the HT method, colonies were confirmed by colony PCR with primers (repA-up/down) after propagation for 100 generations at 42°C. Result showed that with primers (repA-up/down), some colonies amplified low-production DNA fragments but most of them amplified normal production. Colonies with low production were propagated further at 42°C and confirmed by colony

Figure 1. Phylogenetic tree for the replication initiation (Rep) proteins from different plasmids constructed using MEGA 6.0 (BioDesign Institute, Arizona State University, Tempe, AZ). The phylogenetic tree, made using MEGA 6.0, classified the Rep proteins from different plasmids. Color version available online.

Figure 2. (a) Construction of the shuttle vectors pUE-F0, pUE-F1, pUE-F2, pUE-F3, pUE-F4; (b) construction of pUEG-F1-P_{ldh}; (c) pUEG-F1-P_{ldh} and pUEG-F1-P₂₃. Amp = ampicillin; Ori = origin; Em = erythromycin; GFP = green fluorescent protein; Cm = chloramphenicol; orf = open reading frame; pldh and P23 = promoters; LacZ = β-galactosidase; MCS = multiple cloning site; LacR = lac repressor binding site; Plac = promoter of LacZ; and bra = ampicillin. Color version available online.

PCR. When primers (repA-up/down) could not amplify DNA from *L. casei* CP002616 colonies, we obtained positive mutants and named them *L. casei* CP002616 pLC2WΔ-HT (Figure 4a).

For the IC method, the recombinant plasmid pUE-F1 was introduced into *L. casei* CP002616 and positive transformants were confirmed by colony PCR with primers (rmp-up/down). The positive colonies con-

Table 3. Transformation efficiency (cfu/ μ g of DNA) and stability (as plasmid loss rate) of the shuttle vectors in *Lactobacillus casei* CP002616

Item	Plasmid				
	pUE-F0	pUE-F1	pUE-F2	pUE-F3	pUE-F4
Efficiency (cfu/ μ g of DNA)	1.8×10^5	2×10^5	1.3×10^5	8.6×10^5	—
Plasmid loss rate (%)					
After 20 generations	1.96	2.24	2.2	1.98	—
After 40 generations	10.23	9.67	9.5	10.18	—
After 60 generations	35.12	33.87	35.43	35.63	—
After 80 generations	53.89	52.56	54.05	53.91	—

tained 2 plasmids, the cryptic plasmid pLC2W and the (introduced) competitor plasmid pUE-F1. Because of plasmid incompatibility, pUE-F1 substitution and pLC2W elimination could occur in the host cell after several generations under antibiotic pressure. Transformants grew in liquid MRS supplemented with Em for 5 culture passages at least. Transformants were confirmed by colony PCR using rmp-up/down as primers that amplified the special DNA fragment of pLC2W (Figure 4b). Positive colonies were designated *L. casei* CP002616 pUE-F1. Exogenous pUE-F1 was cured by propagating *L. casei* CP002616 pUE-F1 in MRS without antibiotics for 2 culture passages. Exogenous plasmid was unstable without antibiotics, and Em-sensitive clones were considered pUE-F1-cured strains and were named *L. casei* CP002616 pLC2W Δ -IC.

Characterization of EPS and Acid Production

Mutant strain *L. casei* CP002616 pLC2W Δ -HT produced 12% less EPS than the wild-type, whereas the wild-type and IC strains produced similar amounts of EPS (Figure 5a). We proposed that mutations would occur during the process of subculture at high temperature because some mutations will happen during the harsh physical curing process, such as the change of EPS production and delay of acid production.

We analyzed the monosaccharide composition of EPS-HT, EPS-IC, and EPS-WT by HPAEC-PAD. As shown in Table 5, EPS-HT, EPS-IC, and EPS-WT were

mainly composed of rhamnose, arabinose, galactose, glucose, and xylose. The monosaccharide composition was similar among EPS of different strains

The results of high-performance gel-permeation chromatography revealed that EPS-HT, EPS-IC, and EPS-WT exhibited similar elution profiles (data not shown), indicating no significant difference in molecular weight distribution among EPS from mutants and wild type. As shown in Table 5, the molecular weights of EPS-HT, EPS-IC, and EPS-WT were 20.64, 20.58, and 20.62 kDa, respectively.

Figure 5b compares acid production in *L. casei* CP002616 pLC2W Δ -HT, *L. casei* CP002616 pLC2W Δ -IC, and *L. casei* CP002616 (wild type) under normal growth conditions. Acid production was reduced in *L. casei* CP002616 pLC2W Δ -HT, whereas that of *L. casei* CP002616 pLC2W Δ -IC was almost equal to that of the wild type. These results showed that there was no relationship between plasmid and production of EPS or acid.

DISCUSSION

Plasmids are widespread in LAB and usually replicate by RC or theta replication. The biological significance of plasmids in lactobacilli remains unknown but the cryptic plasmids isolated from LAB could be developed as shuttle vectors. *Lactobacillus casei* TISTR1341 have 3 plasmids: pRCEID2.9 with RC replication and pRCEID3.2 and pRCEID13.9 with theta replication. The

Table 4. Transformation efficiency (cfu/ μ g of DNA) and stability (as plasmid loss rate) of pUE-F1 in different hosts

Host strain	Efficiency (cfu/ μ g of DNA)	Plasmid loss rate (%)
<i>Lactobacillus casei</i> CP002616	1.8×10^5	52.56
<i>L. casei</i> BDII	2.2×10^5	65.43
<i>L. casei</i> AR341	1.7×10^5	33.21
<i>Lactobacillus paracasei</i> AR93	8.5×10^4	13.47
<i>Lactococcus lactis</i> NZ9000	1×10^2	—
<i>Lactobacillus plantarum</i> (WCFS1)	—	—
<i>Lactobacillus brevis</i>	—	—

relative copy numbers per chromosome equivalents of pRCEID-LC2.9 and pRCEID-LC13.9 were about 15 and 4, respectively (Panya et al., 2012). Cryptic plasmid pMC11 was isolated from *L. casei* MCJ and has 2 replicons of distinct types. Each replicon is independent and exhibits similar transformation efficiencies and copy numbers. Both replicons replicate via theta replication (Chen et al., 2014). All these cryptic plasmids

from LAB were developed to be the shuttle vectors or expression vectors used in LAB.

Here, we studied the cryptic plasmid pLC2W from *L. casei* CP002616, which can produce 3 types of extracellular polysaccharides (Ai, 2008) and has been sequenced previously. In this report, we compared the AA sequence of the Rep protein of pLC2W with those of theta-type and RC-type plasmids in *Lactobacillus*. The RepA protein of pLC2W belonged to the RC replication group, based on phylogenetic analysis. Therefore, the replicon of pLC2W would replicate via RC replication. Nucleotide sequence analysis revealed 4 putative ORF related to replication, and we determined that the shortest functional replicon of pLC2W contained the origin region and repA.

The new shuttle vectors pUE-F1 developed in this work were shown to replicate in different lactobacilli with a transformation efficiency of 10^2 to 10^5 cfu/ μ g. This high efficiency made pUE-F1 useful in developing genetic tools in those lactobacilli. To construct an expression vector, we chose P₂₃, a strong promoter that has been used in LAB, and P_{ldh}, a strong promoter from *L. casei* CP002616.

Lactobacillus casei CP002616 is an EPS-producing strain and it can help to lower blood pressure (Ai, 2008). Some reports have described the involvement of plasmids in EPS biosynthesis in LAB (Vedamuthu and Neville, 1986; von Wright and Tynkkynen, 1987; Neve et al., 1988). Plasmid pNZ4000 is essential for EPS biosynthesis of *Lactococcus lactis* (van Kranenburg et al., 1997); however, no evidence indicated a similar result in *L. casei*. Thus, plasmids are essential for EPS synthesis in some but not all bacteria. Sequence analysis of pLC2W showed that there were no ORF involved in EPS synthesis. We speculated there was no relationship between plasmid and EPS production in *L. casei* CP002616. To prove this hypothesis, we implemented 2 plasmid-curing methods. Several methods have been developed for plasmid curing in different strains, and physical methods are widely used. A cryptic plasmid in *Streptococcus lactis* was cured by culture at subinhibitory temperature (Gasson and Davies, 1980; Heery et al., 1989). Some chemical methods have been used to eliminate plasmids. For example, plasmid from *Streptomyces* was cured using the DNA intercalating agent ethidium bromide, and plasmid-free mutants were obtained (Crameri et al., 1986). Meanwhile, a sequential protoplast generation-recovery process was used to eliminate plasmid pFP₄ from *Streptomyces* FR1 (Chen et al., 2012). Some reports indicate that traditional curing methods can introduce mutational events in strains at high frequency, and these mutations may interfere with the functional analysis of the plasmid (Poppe and

Figure 3. Photograph of cells of lactobacilli species, with fluorescence microscopy (left) and phase contrast microscopy (right) of (a) *Lactobacillus casei* CP002616, and (b) *Lactococcus lactis* NZ9000, and (c) green fluorescent protein (GFP) expression (measured as optical density ratio at 480 and 600 nm, OD_{480/600}) of plasmids pUEG-F1-P_{ldh}, pUEG-F1-P₂₃, and pIB184-GFP. Error bars indicate standard deviations. Color version available online.

Figure 4. Identification by PCR of the plasmid-cured mutants: (a) high-temperature (HT) method: lanes 1 to 23 = colonies and lane 24 = wild-type strain (control); and lane M = molecular weight marker. The amplified 1.2-kb fragment corresponded to the plasmid-specific gene; (b) plasmid incompatibility (IC) method: lanes 1 to 15 = colonies; lane 16 = wild-type strain (control); and lane M = molecular weight marker. The amplified 0.8-kb fragment corresponded to the plasmid-specific gene on plasmid pLC2W.

Gyles, 1988). Plasmid incompatibility was developed as a milder strategy to cure cryptic plasmid. In this study, we used 2 curing methods (HT and IC) to eliminate the native plasmid pLC2W from *L. casei* CP002616.

Analysis of monosaccharide compositions and molecular weights of EPS-HT, EPS-IC, and EPS-WT showed no difference between them. Production of EPS and acid were reduced in *L. casei* CP002616 pLC2WΔ-HT, whereas the plasmid-cured strain *L. casei* CP002616 pLC2WΔ-IC showed EPS and acid production similar to that of the wild type. An unexpected mutation might have occurred during the HT plasmid-curing process. The HT method is also labor intensive and

time consuming, whereas the IC method is rapid and highly efficient.

In conclusion, we characterized the replication of the novel plasmid pLC2W and constructed a new shuttle vector based on its replication. In addition to the origin region on pLC2W, the promoter, GFP, and multiple cloning site (MCS) were added to construct the new expression vectors pUEG-F1-P₂₃ and pUEG-F1-P_{ldh}, which could serve as a reporting system in other lactobacilli. In addition, we developed a smaller plasmid pUE-F1 with the same replicon to obtain plasmid-free strains of *L. casei* CP002616 via the plasmid incompatibility curing method. Sequence analysis of pLC2W and plasmid-curing results showed that plasmid pLC2W is not involved in EPS synthesis.

Table 5. Monosaccharide compositions (molar ratio) and molecular weight (MW) of exopolysaccharide (EPS) produced by strains *Lactobacillus casei* CP002616 pLC2WΔ-HT (plasma cured by heat treatment, HT), *L. casei* CP002616 pLC2WΔ-IC (plasma cured by incompatibility, IC), and *L. casei* CP002616 (wild-type, WT), respectively

Item	EPS-HT	EPS-IC	EPS-WT
Molar ratio			
Rhamnose	3.2	3.1	3.3
Arabinose	1.0	1.2	0.9
Galactose	3.2	3.3	3.0
Glucose	2.6	2.5	2.6
Xylose	0.1	0.1	0.2
MW (kDa)	20.64	20.58	20.62

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Figure 5. Differences in (a) exopolysaccharide (EPS) production and (b) acid production between the wild-type strain and pLC2WΔ-IC and pLC2WΔ-HT (plasmid-cured) mutant strains. Error bars indicate standard deviations. Color version available online.

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