RESEARCH ARTICLE

Isolation of a minireplicon of the plasmid pG6303 of *Lactobacillus plantarum* G63 and characterization of the plasmid-encoded Rep replication protein

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Abstract

A cryptic 10.0-kb plasmid pG6303 from a multiplasmid-containing *Lactobacillus plantarum* G63 was studied. The analysis of replicon was facilitated by the construction of shuttle vectors and electrotransformation into *L. plantarum*. The pG6303 replicon included (i) an open reading frame encoding the putative Rep replication initiation protein; and (ii) the putative origin of replication. The Rep protein was expressed as a fusion with the hexa-histidine (His) at its C-terminal end and purified by Ni-affinity chromatography. The electrophoretic mobility shift assays in pG6303 showed that the purified Rep protein specifically bound from 5582 to 5945 bp, differing from the putative origin of replication of pG6303. We speculate that pG6303 replication is a new mode of plasmid replication.

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Introduction

Lactic acid bacteria (LAB) is the generic name of bacteria that produce high levels of lactic acid by fermentation of various sugars and carbohydrates (Wada *et al.* 2009). The common species are *Lactobacillus acidophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. casei*, *L. helveticus*, *L. fermentum*, *L. plantarum* and *L. reuteri*. *L. plantarum* is one of the most important *Lactobacilli*, and is often used in grass silage, meat and vegetable fermentation (Xi *et al.* 2013). Hence, genetic engineering techniques have been used to improve desired characteristics of LAB.

L. plantarum species often harbours several plasmids which range from 2.0 to 68 kb in size (Ruizbarba *et al*. 1991; Xi *et al*. 2013). The circular plasmids in *L. plantarum* replicate in two modes, the rolling-circle replication (RCR, <12 kb), such as pA1, pWCFS101 and pWCFS102 (Vujcic and Topisirovic 1993; van Kranenburg *et al*. 2005b). The replicons of RCR plasmids consist of replication initiator proteins (Rep) and replication origin (OriV). The OriV is made up of the double strand origin of replication (*dso*) and single-stranded (*sso*) (Khan 2005). The *dso* is highly specific for its cognate Rep and contain domains that are involved in the initiation as well as termination of leading-strand replication (Khan 1997). The function of *sso* is to support replication of the lagging strand (Tanaka *et al.* 2005). The other mode is theta replication, such as pMD5057, pRV500 and p256 (Danielsen 2002; Alpert *et al.* 2003; Sorvig 2005). The theta replicons can be classified according to their dependency on three key components, Rep proteins, OriV and host-encoded DNA polymerase I (Alpert *et al.* 2003). Rep proteins and OriVin theta replication have similar function as that of rolling-circle replication (RCR), while the host-encoded DNA polymerase is to synthesize nascent strand DNA. Hence, both modes of replication need Rep and OriV.

Previously, we found that there were three plasmids designated pG6301(3516-bp), pG6302(9112-bp) and pG6303(10047-bp) in *L. plantarum* G63 (Xi *et al.* 2013). However, we neither found OriV nor Rep protein in pG6303 plasmid via DNA and protein sequence analysis. In this study, we investigated the minimal replicon and Rep protein of pG6303, and expressed and purified the Rep protein.

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Keywords. cryptic plasmid; replicon; Rep protein; *Lactobacillus plantarum.*

Materials and methods

Bacterial strains and culture conditions

L. plantarum G63 was grown under anaerobic conditions at 37°C in MRS medium (McLaughlin 1946). *Escherichia coli* DH10B and *E. coli* BL21(DE3) were grown aerobically with shaking at 180 rpm at 37°C in Luria–Bertani (LB) medium (Sambrook *et al.* 1989). For the cloning procedures *E. coli* DH10B was used as a cloning host. Proteins were overexpressed in *E. coli* BL21(DE3). Antibiotics were added to the medium as and when necessary at the following final concentrations: ampicillin 100 μg/mL, kanamycin 50 μg/mL and chloramphenicol 10 μg/mL.

Plasmid DNA isolation and DNA manipulations

Plasmid DNA was isolated from *E. coli* using the BioTeKe plasmid extraction kit (BioTeKe, Beijing, China) according to the manufacturer's instructions. Plasmid DNA was isolated from *L. plantarum* by using the alkaline lysis method with some modifications. The experiments steps were as follows. Cultures of *L. plantarum* grown to OD 600 0.8–1.0 were collected by centrifugation and washed in TE buffer (50 mM Tris–HCl, 30 mM EDTA, pH 8.0). Lysozyme was then added at a final concentration of 30 mg/mL, and the suspension was incubated at 37°C for 1 h. Total plasmid DNA from *L. plantarum* was prepared as previously described (Te Riele *et al.* 1986).

Plasmids construction and transformation procedures

To identify the pG6303 minimal replicon, several recombinant plasmids were constructed in *E. coli* DH10B and, subsequently, electroporated into *L. plantarum* G63. Inserts were obtained by PCR amplification of the pG6303 region using *L. plantarum* G63 plasmids as template with the 1F/1R, 2F/2R, 3F/3R, 4F/4R, 5F/5R, 6F/6R, 3F/31R, 32F/32R, 33F/3R, 3F/34R, 35F/31R, 36F/31R, 37F/31R, 33F/38R, 33F/39R and 3F/310R primer pairs (table 1). The amplified fragments were then cloned into TA clone vector pMD19-T simple (Takara, Dalian, China). Then, the chloramphenicol resistant gene with intact promoter region was amplified by PCR using plasmid pNZ8048 as template with CmF/CmR primer pair (table 1). The amplified DNA fragment was cloned into TA clone vector pMD19-T simple and digested with *Sca*I and *Aat*II. The recovered chloramphenicol resistance gene was inserted into the *Sca*I and *Aat*II of vector pMD19-T simple containing pG6303 regions, resulting in pUC63031, pUC63032, pUC63033, pUC63034, pUC63035, pUC63036, pUC63037, pUC63038, pUC63039, pUC630310, pUC630311, pUC630312, pUC630313, pUC630314, pUC630315 and pUC630316, respectively.

To verify the minimal replicon of pG6303, the above constructed plasmids were tested by electroporating into the *L. plantarum* G63 and *L. plantarum* NC8. Electrocompetent cells were prepared and transformed with the pG6303 derivative plasmids according to Aukrust and Blom (1992) with some modifications. Precultures of *Lactobacillus* were obtained in MRS medium incubated at 37°C overnight. Competent cultures were obtained after inoculating 500 μL of the preculture to 50 mL MRS medium containing 1% glycine, and further incubated at 37°C. When OD_{600} reached 0.8–1.0, strain growth was stopped and harvested at 4°C. Cells were washed with 1 mM $MgCl_2$ and 30% PEG1000, suspended 500 μL 30% PEG1000. Immediately before electroporation, 5 μL DNA solution (20 μg plasmid DNA) was added per 80 μL cell suspension. The mixture was placed into a 0.2 cm electroporation cuvette using a Gene Pulser apparatus (Biorad, Richmond, USA). After pulsing under specific conditions (field strength, 1500 V; capacitance, 25 mF; resistance, 400 V), the cell suspension was immediately diluted with 800 μL MRS broth and incubated at 37°C for 2 h. The cell

Table 1. PCR primers used in this study.

Name	Sequence (5′–3′)
1F	CCAGGAAAATGGGAAGAT
1R	CCGAGTGAAACGAGGTC
2F	GTAACCGAATAGGGCGC
2R	TCTGAACAGCAAAGGGC
3F	ACGGGACGGCTAGTACAT
3R	AAAACAACCCCAGAACG
4F	CCGTGTTGGTAAAGGTCT
4R	GTTGCTGTCTCTGGTAAAG
5F	GCCTGGTCTTCTACTTGT
5R	CTACTCGAACGTGTGTTC
6F	TGAGAAAAAAAACAACTAAT
6R	TTGATGACAAATAGAAGAAG
31R	AAGGGCTTATTCGGCTGGCT
32F	CTGTCAGTCCCCGAAAGGT
32R	AGACGTGTTCTAAGCCCTCG
33F	TACCCCTTTGCCGATAAT
34R	GGGGGAACACGTAAATCAT
35F	CTTGAACCTGAGCGGGAT
36F	GCACTTGCCCTTTGCTGT
37F	TCACGAATCACCGTCACG
38R	TCAGGCAGAGCTTGGGTC
39R	TTGGATATTGGACAGCCC
310R	GATTCAGGCAGAGCTTGG
CmF	**AGTACT**TATAGAGCAAGTTATGCAAAGG[a]
CmR	**GACGTC**TACAGTCGGCATTATCTCATAT[b]
RepF	**CTCGAG**GCCAGCCGAATAAGCCCTT[c]
RepR	**CATATG**GCCCTCGTTCAGTGTTGA[d]
M13F	CGCCAGGGTTTTCCCAGTCACGAC
M13R	AGCGGATAACAATTTCACACAGGA
5945R	ATCGGTAGGCTTATGGTA
5332F	TTCTTTCGTTCAAGTTCG
5582F	ATAATCGTTCATTGTTGTTC
5664F	AACGAGGGCTTAGAACAC
5672R	GCCCTCGTTCAGTGTTGA
5856R	TTCGTTCGGAATAGGTTA
5793R	ACCATTTTAAAGGAAGCT
5776F	AGCTTCCTTTAAAATGGT

[a]Boldface type indicates the *Sca*I site; [b]boldface type indicates the *Aat*II site; [c]boldface type indicates the *Xho*I site. [d]boldface type indicates the *Nde*I site.

suspension was placed on selective media and incubated at 37°C for 3–4 days. Finally, the clones were tested by the isolation of plasmids and PCR.

Methods for plasmid elimination

To future verify the replicon of pG6303, we used acridine orange as elimination agent to eliminate plasmid in *L. plantarum* G63. Single colony of *L. plantarum* G63 was inoculated to 3 mL MRS medium without antibiotics. Then, cells were incubated at 37°C overnight. Consequently, 30 μL of the cells were inoculated in 3 mL MRS medium containing 20 μg/mL acridine orange at 37°C for 24 h.

After five generations, a portion of the cell suspension was placed on MRS media and incubated at 37°C for three days. Finally, the single clones were inoculated to 3 mL MRS and tested by the isolation of plasmids and PCR.

RT-PCR

The total RNA concentration was determined spectrophotometrically at 260 nm and 100 ng was used for reverse transcription using the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) according to the manufacturer's instructions, the reaction system which contained 1 μL random hexamer primer (0.5 μg/μL), 5μL RNA (20 ng/μL) and 6 μL DEPC was incubated at 65°C for 5 min. Consequently, 4 μL 5× reaction buffer, 1 μL Ribolock RNase inhibitor (20 U/μL), 2 μL 10 mMdNTP mix and 1 μL reverse transcriptase (200 U/μL) added to the reaction system above. Then the mixture was incubated at 25°C for 5 min, at 42°C for 60 min and at 70°C for 5 min in an order. Following that, the first strand cDNA of *L. plantarum* was acquired. Next, the *rep* gene from plasmid pG6303 was amplified using the first-strand cDNA as template with the RepF/RepR primer pair (table 1). The resulting PCR products were analysed on a 1.0% agarose gel. The *rep* gene from plasmid pG6301 was also reversely transcribed and amplified as a positive control. The plasmids of *L. plantarum* were also used as the template for a positive control.

Plasmid incompatibility analysis

For incompatibility analysis, *L. plantarum* G63 containing different recombinant plasmid were passaged. Single colony of *L. plantarum* G63 containing recombinant plasmid was inoculated to 3 mL MRS medium without antibiotics, and incubated at 37°C. Inoculate *L. plantarum* G63 cells into new 3 mL MRS medium every 24 h. After five generations, a small portion of the cell suspension was placed on MRS media and incubated at 37°C for three days. The methods of verification are described above.

Protein expression and purification of Rep protein

The *rep* gene of plasmid pG6303 was obtained by PCR amplification using *L. plantarum* G63 plasmids as template with RepF/RepR primer pair (table 1). The amplified DNA fragment was cloned into TA clone vector pMD19-T simple and digested with *Nde*I and *Xho*I, inserted into the same sites of expression vector pET29a (Invitrogen Carlsbad, USA), resulting in pET29a-6303Rep plasmid. Rep protein expression in *E. coli* BL21(DE3) was carried out at 16°C for 24 h. Once the OD_{600} of the culture fluid reached 0.5–0.6, IPTG was added to a final concentration of 0.2 mM to induce the expression of Rep. Recombinant Rep protein was expressed as 6× His-tagged in C-terminal for affinity purification. Procedures for purification of the Rep protein were as described by Kwong *et al.* (2001). The sequences of Rep protein from the plasmid DNA were compared to those present in the GenBank using BLAST (Altschul *et al.* 1997). The phylogenetic analysis of Rep protein sequences was done using the neighbour-joining method and the MEGA 4.1 (Tamura *et al.* 2007). The statistical significance of the grouping was estimated by bootstrapping (1200 replicates).

Electrophoretic mobility shift assays (EMSA)

A series of DNA fragments from pG6303 were PCR amplified using primers 3F/3R, 31F/31R, 33F/3R, 33F/5945R, 5332F/3R, 33F/5672R, 5582F/3R, 5332F/5945R, 5582F/5945, 5664F/5945R, 5582F/5856R, 5582F/5793R, 5776F/5945R, 5664F/5856R, designated P101, P102, P103, P104, P105, P106, P107, P108, P109, P110, P111, P112, P113 and P114. EMSA was performed essentially as described previously, with some modifications (Kwong *et al.* 2004). Briefly, DNA fragment was incubated with purified Rep protein in binding buffer (27.5 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.075 mM EDTA, 0.5 mM dithiothreitol, 0.05% Triton X-100, 10% glycerol) and incubated on 20°C for 30 min. Binding reactions were separated on 1% agarose gel.

Results and discussion

Bioinformatics analysis of pG6303

L. plantarum G63 strain harbours three plasmids pG6301, pG6302 and pG6303 (Xi *et al.* 2013). The complete DNA sequence of pG6303 (GenBank accession number JX174169) is 10047 bp in length. The average G + C content of pG6303 is 36.8%, which is slightly below and above the typical G + C content of 39.5 and 34.3% reported for pWCFS101 and pWCFS102, respectively (van Kranenburg *et al.* 2005a). Plasmid pG6303 harbours nine putative protein encoding ORFs of more than 120 nt in length (Xi *et al.* 2013). However, neither *rep* gene nor typical replication initiating region can be found in pG6303 based on GenBank database homology searching.

To predict the putative OriV, GC skew index was calculated for pG6303 (Arakawa *et al.* 2009). The replication initiating region of pG6303 was predicted with GenSkew (http://genskew.csb.univie.ac.at/). This method is based on the general observation that GC content usually differs between the leading and lagging strands of replication forks (Lobry 1996;

Soler *et al.* 2010). Strikingly, the minimal and maximal of cumulative GC skews of pG6303 are located between 4801 and 8101 bp in pG6303, respectively. We speculate that one of these two regions is the replication initiation region of pG6303.

Determination of rep gene of pG6303

To identify the potential replication initiation region of pG6303, six shuttle vectors pUC63031, pUC63032, pUC63033, pUC63034, pUC63035 and pUC63036 based on the *orf*s of pG6303 were constructed (table 2). Since ampicillin resistance gene can reduce the efficiency of electroporation, chloramphenicol-resistance gene and its promoter from pNZ8048 replaced a part of ampicillin resistance gene from vector pMD19-T simple (Tanaka *et al.* 2005). All the six shuttle vectors were introduced into the *L. plantarum* G63 and the plasmid-free *L. plantarum* NC8 using electroporation and selected for chloramphenicol resistance (table 2). pUC19 and pNZ8048 were used as negative and positive control, respectively. The presence of the recombinant plasmids in the transformed clones was verified by plasmid extraction and PCR amplification of the inserts using M13F/M13R primer pair (table 1). The results showed that only pUC63033 can replicate in *L. plantarum* G63 and *L. plantarum* NC8 in all five experiments. The inserted fragment of pUC63033 ranging from 4356 to 6160 bp in pG6303 is sufficient to support replication in *L. plantarum*, indicating that both *rep* gene and OriV are possibly included in this fragment. However, the only *orf* in this region is *orf*6 and *orf*6 shares a high degree of similarity with plasmid pLP9000 and Lp16L that have no *rep* gene identified. The protein encoded by *orf*6 shares 95% identity with the hypothetical protein (NP_631992) of pLP9000 of *L. plantarum* AS1.2986 (Daming *et al.* 2003) and 90% identity with the hypothetical protein (AGO09800) of Lp16L of *L. plantarum* 16 (Pappin *et al*. 1993; Crowley *et al*. 2013) (figure 1a). Further, the promoter of *orf*6 is typical mode of promoter and own the same −10 box, −35 box and ribosome-binding site (RBS) as the hypothetical protein of pLP9000 and Lp16L (figure 1b). In conclusion, according to the results of our electroporation experiments and sequence analysis above, we deduced that these *orf*s in these three plasmids (pG6303, Lp16L and pLP9000) have the same function and are the putative *rep* gene of these three plasmids.

Determination of the minimal replicon of pG6303

Subcloning of the insert fragment of pUC63033 was carried out to localize the pG6303 minimal replicon. The recombinant vectors pUC63037, pUC63038, pUC63039,

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristic	Reference or source
Strains		
E. coli		
DH 10B	F⁻mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galUgalK λ⁻rpsLnupG	Invitrogen
BL21(DE3)	F- ompThsdS($r_B^- m_B^-$) gal dcm(DE3)	Invitrogen
L. plantarum		
G63	Wild-type strain; harbours pG6301, pG6302 and pG6303	Xi *et al.* (2013)
G631	Harbours pG6301 and pG6302	This study
G632	Harbours pG6301, pG6302 and pUC63033	This study
G633	Harbours pG6301, pG6302, pG6303 and pUC63033	This study
NC8	Wild-type strain, plasmid-free	Aukrust and Blom (1992)
Plasmids		
pUC63031	Cm^r; 2458-bp fragments of pG6303(27-2512 bp) subcloned in pMD19-T simple	This study
pUC63032	Cm^r; 2222-bp fragments of pG6303(2246-4468 bp) subcloned in pMD19-T simple	This study
pUC63033	Cm^r; 1804-bp fragments of pG6303(4356-6160 bp) subcloned in pMD19-T simple	This study
pUC63034	Cm^r; 2337-bp fragments of pG6303(6090-8427 bp) subcloned in pMD19-T simple	This study
pUC63035	Cm^r; 1677-bp fragments of pG6303(8324-10001 bp) subcloned in pMD19-T simple	This study
pUC63036	Cm^r; 2813-bp fragments of pG6303(7424-190C bp) subcloned in pMD19-T simple	This study
pUC63037	Cm^r; 774-bp fragments of pG6303(4356-5129 bp) subcloned in pMD19-T simple	This study
pUC63038	Cm^r; 846-bp fragments of pG6303(4840-5685 bp) subcloned in pMD19-T simple	This study
pUC63039	Cm^r; 1095-bp fragments of pG6303(5065-6160 bp) subcloned in pMD19-T simple	This study
pUC630310	Cm^r; 653-bp fragments of pG6303(4356-5008 bp) subcloned in pMD19-T simple	This study
pUC630311	Cm^r; 658-bp fragments of pG6303(4472-5129 bp) subcloned in pMD19-T simple	This study
pUC630312	Cm^r; 684-bp fragments of pG6303(4446-5129 bp) subcloned in pMD19-T simple	This study
pUC630313	Cm^r; 594-bp fragments of pG6303(4536-5129 bp) subcloned in pMD19-T simple	This study
pUC630314	Cm^r; 935-bp fragments of pG6303(5065-5999 bp) subcloned in pMD19-T simple	This study
pUC630315	Cm^r; 836-bp fragments of pG6303(5065-5900 bp) subcloned in pMD19-T simple	This study
pUC630316	Cm^r; 1647-bp fragments of pG6303(4356-6002 bp) subcloned in pMD19-T simple	This study
pNZ8048	Cm^r; high-copy-number *E. coli-L. lactis* overexpression vector, PnisA	O'Driscoll *et al.* (2006)
pET29a-6303Rep	Expression vector	This study

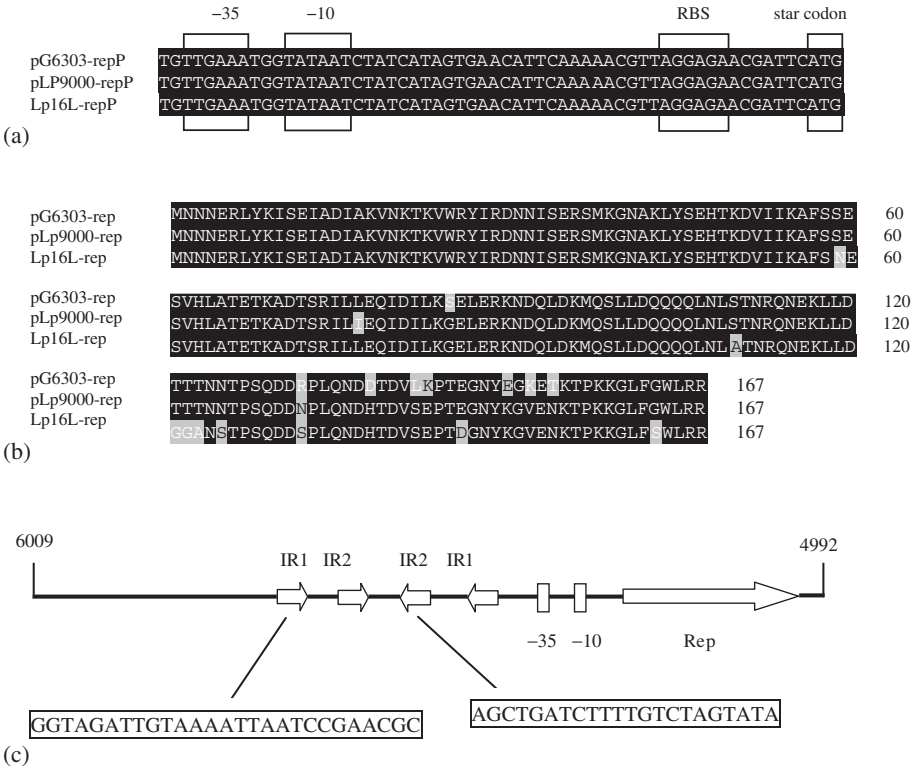

Figure 1. (a) Promoter, (b) alignment of the Rep protein of pG6303, pLp9000 and Lp16L. The alignment was done using the Bioedit program, and the shaded letters indicate identical amino acids or nucleotides. (c) Structural organization of the region of pG6303. The −35 and −10 boxes of the *rep* gene are represented. Each unit of identified inverted (IR) repeats is depicted by an arrow. The sequence of the repeat units is indicated below.

pUC630310, pUC630311, pUC630312, pUC630313, pUC630314, pUC630315 and pUC630316 were constructed and electroporated into *L. plantarum* G63 and *L. plantarum* NC8, respectively (table 2). Their ability to maintain replication in these strains is depicted in figure 2. Results showed that pUC63037 and pUC630316 could replicate in *L. plantarum* G63, while only pUC630316 could replicate in *L. plantarum* NC8. We deduced that pG6303 could provide Rep protein to satisfy pUC63037 replication in *L. plantarum* G63, while in *L. plantarum* NC8, there was no suitable Rep

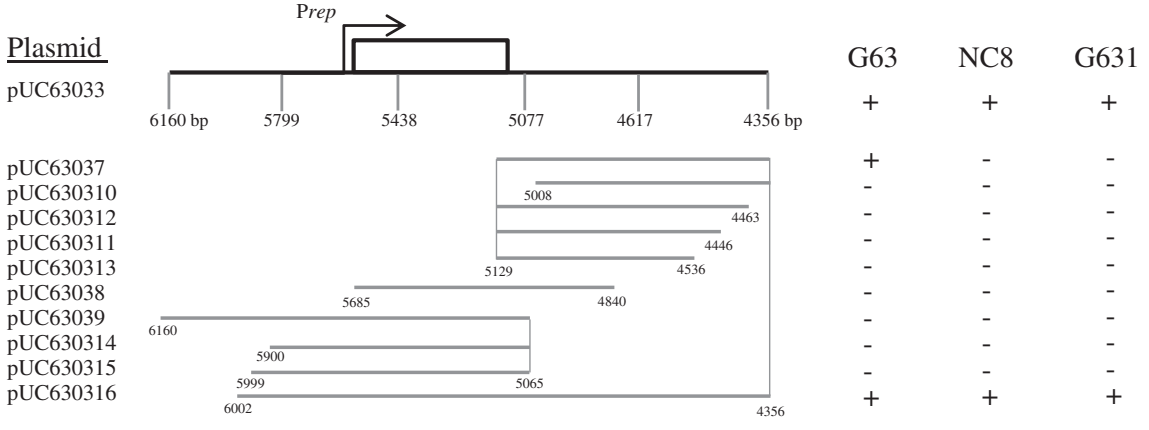

Figure 2. Physical map of the pG6303 replication region and deletion derivatives. Plasmid pUC63033 contains the pG6303 replication region from coordinates 6160 to 4356. The *rep* coding region (white box) gives rise to the replication initiation protein. The positions of the *rep* promoters (P_{rep}) are indicated by arrows. Grey bars indicate the extent and position of fragments carried by deletion plasmids that were generated to localize essential regions. The three columns on the right indicates each plasmid's ability (+) or inability (−) to replicate in *L. plantarum* G63, *L. plantarum* NC8 and *L. plantarum* G631. Each experiment was repeated five times.

protein to support pUC63037 replication (Iordanescu 1989; Wang *et al.* 1992). Since deletion from each side of the fragment caused the failure of replication initiation, we could summarize that the fragments which located from 4356 to 5129 bp in pG6303 is the OriV of pG6303. The length of the fragment is 774 bp. Interestingly, most of the plasmids which replicate in RCR or theta type have direct repeats (DRs) or inverted repeats (IRs) in their OriV region, such as pMRI5.2, pLS1 and pDOJH10L (Moscoso *et al.* 1995; Lee and O'Sullivan 2006; Cho *et al.* 2013). We actually found two IRs in the upstream of the promoter of *rep* gene of pG6303. But these two IRs were not in this 774 bp fragment (figure 1c). However, we did not get any useful information when the 774 bp fragment was aligned in GenBank. The overall AT content of the 774 bp fragment was 56% which was even lower than that of pG6303. But AT-rich region could be detected in this fragment. AT content of 671–711 region of the 774-bp fragment amounted to 85.4%. Having AT-rich region was one the distinctive character of replication initiation sites (del Solar *et al.* 1998). Interon structures were not found in this fragment. Usually, interons were important for plasmid replication. Several exceptions were reported to replicate without interon structure (del Solar *et al.* 1998). But pG6303 shares no similarity with these plasmids.

As mentioned above, pG6303, pLP9000 and Lp16L have similar putative Rep proteingene, we suppose they own the same replication mode and OriV. ClusterW alignment showed that the 774-bp fragment shares 97% and 96% similarity with corresponding regions of pLP9000 and Lp16L, respectively. These results indicate that pG6303, pLP9000 and Lp16L belong to a new plasmid family with novel replication mechanism.

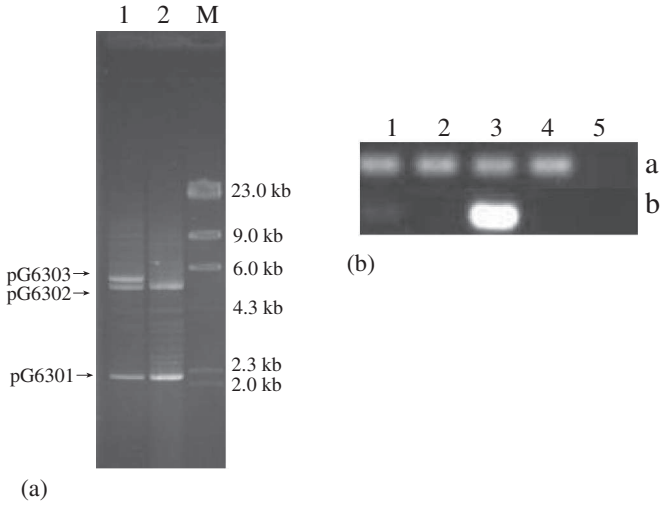

Figure 3. Agarose gel electrophoresis of plasmids from *L. plantarum* G63 and G631. Plasmid DNA was isolated by the alkaline lysis method with some modifications and DNA was electrophoresed in a 0.70% (w/v) agarose gel. Three circular plasmids are indicated by arrowheads. Lane 1, plasmids from *L. plantarum* G63; lane 2, plasmids from *L. plantarum* G631; lane M, DNA ladder marker. (B) PCR product bands of the *rep* from pG6301 (a) and *rep* from pG6303 (b). The PCR templates were Lane 1, cDNA of *L. plantarum* G63; lane 2, cDNA of*L. plantarum* G631; lane 3, plasmids extracted from *L. plantarum* G63; lane 4, plasmids extracted from *L. plantarum* G631; lane 5, sterile water as a negative control.

Plasmid elimination and passage experiment

According to the above results, we found that the fragment which range from 4356 to 6002 bp in pG6303 can support the replication of pUC630316, and Rep expressed from pG6303 can support the replication of pUC63037 (minimal replicon without *rep*) in *L. plantarum* G63. However, we cannot determine which Rep protein could support the replication of pUC63033 in *L. plantarum* G63, the one from pUC63033 or that from pG6303. We eliminated pG6303 by acridine orange in *L. plantarum* G63 and acquired *L. plantarum* G631 which harbors only pG6301 and pG6302 (figure 3a). The elimination of pG6303 was verified by plasmid isolation and detecting the expression of *rep* in *L. plantarum* G63 and G631 by RT-PCR. As shown in the figure 3b, we found that *L. plantarum* G63 could express the *rep* gene from pG6303, while no expression of *rep* was detected in *L. plantarum* G631. Results verified that pG6303 was eliminated in *L. plantarum* G631. Then, all shuttle vectors that we construct in this study (table 2) are electroporated into *L. plantarum* G631. pUC19 and pNZ8048 were used as negative control and positive control, respectively. The results are in good agreement with our speculation (figure 2). Only pUC63033 and pUC630316 can replicate in *L. plantarum* G631. pUC63037 cannot be electroporated into *L. plantarum* G631 because of lack of Rep protein to support its replication. Open reading frame analysis showed that there is only one *orforf*6 in 4356–6002 bp. These results further verified that *orf6* is the *rep* gene of pG6303.

Two genetically distinguishable plasmids that cannot be stably maintained within a particular host are designated as members of the same incompatibility group (Datta 1979). And the phenomenon of plasmid incompatibility is a consequence of two plasmids sharing common elements responsible for plasmid maintenance, namely, replication control and/or partitioning systems (Kittell and Helinski 1993). Theoretically, pUC63033 and pG6303 have the same replication system, they cannot coexist in *L. plantarum* G63. We used

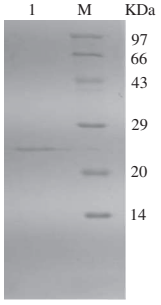

Figure 4. Purification of the Rep protein of pG6303. Lane 1, purified Rep; lane M, protein molecular mass standards (in kDa).

L. plantarum G633 that contains plasmids pG6301, pG6302, pG6303 and pUC63033 to conduct passage experiments as described in materials and methods. After four generations of passage we got *L. plantarum* G632 which contains pG6301, pG6302 and pUC63033.

Based on the above results, we can conclude that (i) the fragment in pUC63033 from pG6303 is the replicon of pG6303 and contains both *rep* gene and OriV; and (ii) the fragment of pUC63037 (4356–5129 bp) from pG6303 is the OriV of pG6303.

Overexpression and purification of the Rep protein

To further analyse the relationship between the Rep and OriV, the Rep protein was overexpressed and purified by affinity purification on a Ni-affinity column. The purity of the protein was tested by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis and staining with Coomassie brilliant blue. The purified Rep protein that fused 6× His is 20 kDa. However, the band of Rep protein is about 25 kDa in SDS-PAGE which is bigger than 20 kDa, and the Rep protein sequencing is correct after peptide massfingerprinting (Pappin *et al.* 1993) (figure 4). So we confirm the Rep protein is correct and can continue to our experiments.

L. plantarum strains harbour one or more natural plasmids (Ruizbarba *et al.* 1991). Some of them replicate via a RC mechanism, while others replicate via the theta mechanism (Yin *et al.* 2008). RC plasmids can be classified into at least five families based on identity in their Rep protein (Khan 1997). The typical plasmids of these five families are pT181, pE194, pC194, pSN2 and pIJ101, respectively (Khan and Novick 1982; Kendall and Cohen 1988; Iordanescu 1989; Novick 1989). On the other hand, at least three classes of theta Rep protein have been recognized, such as that of pSC101, pAMβ1 and pCI2000 (Bruand *et al.* 1993; Meijer *et al.* 1995; Kearney *et al.* 2000). Phylogenetic tree was constructed based on the Rep amino acid sequences of various plasmids that typically replicated by RCR or theta replication exhibited identity to these proteins from pG6303, Lp16L and pLp9000 using the NJ method (Khan 1997; Cho *et al.* 2013) (figure 5). Previous studies showed that plasmids pWV01, pE194, pUB110, pT181, pC221, pJV1, pSN22, pIJ101 and pC194 replicate via RC mechanism and plasmids pAMβ1, pSM19305, pWV02, pSC101, pCI2000 and pLS32 replicate via theta mechanism (Bruand *et al.* 1993; Khan 1997). The resulting phylogenetic tree showed that (i) the Rep proteins from pG6303, pLp9000 and Lp16L are clustered together to form a dependent branch; and (ii) the Rep protein of pG6303 does not belong to any known Rep family of RC or theta plasmids. We conclude that the Rep proteins of plasmid pG6303, Lp16L and pLp9000 are a new family of replication protein of plasmid.

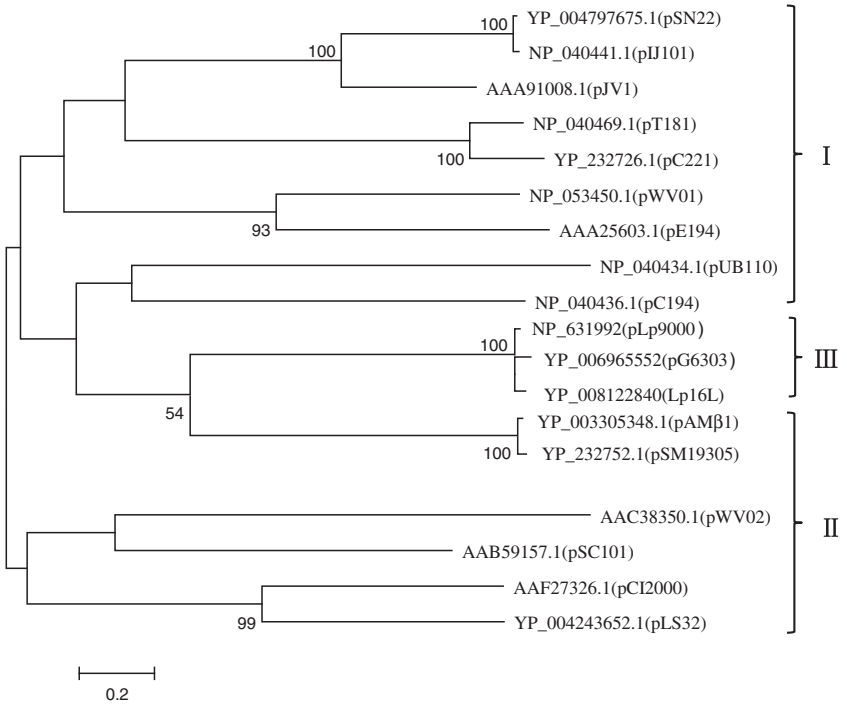

Figure 5. Phylogenetic tree of Rep amino acid sequences of pG6303 with Rep amino acid sequences from plasmids obtained from the GenBank database reconstructed using the neighbour-joining method (1200 replicates). The numbers indicate bootstrap values for branch points. I, plasmids replicate by RCR; II, plasmids replicate by theta replication; III, plasmids pG6303, pLp9000 and LpL16L.

Binding of Rep protein and pG6303 regions

To analyse the interaction between pG6303 and its Rep protein, we tested the specificity of Rep protein binding to DNA using EMSA experiments. P101 (4356–6160 bp), P102 (4356–5129 bp) and P103 (5065–6160 bp) were selected to perform EMSA experiments as described in materials and methods (figure 6a). In contrast to the results of electroporation, Rep protein can bind to P103 which cannot support pG6303 replication in electroporation experiments, while it cannot bind P102 on which the putative OriV locates.

To get the detailed information on the range of Rep protein binding, we further used P104–P114 to perform EMSA experiments (figure 6, b, c&d). The results of our studies demonstrated that only P104, P105, P107, P108 and P109 could bind Rep protein from pG6303 and the minimal region of Rep protein binds locates from 5582 to 5945 bp in pG6303.

RC plasmids need Rep proteins, *dso* and *sso* to start their replication (Khan 1997). A typical example of RC plasmid is plasmid pT181 which has IR sequences in its OriV, and EMSA experiments showed that the Rep protein from pT181

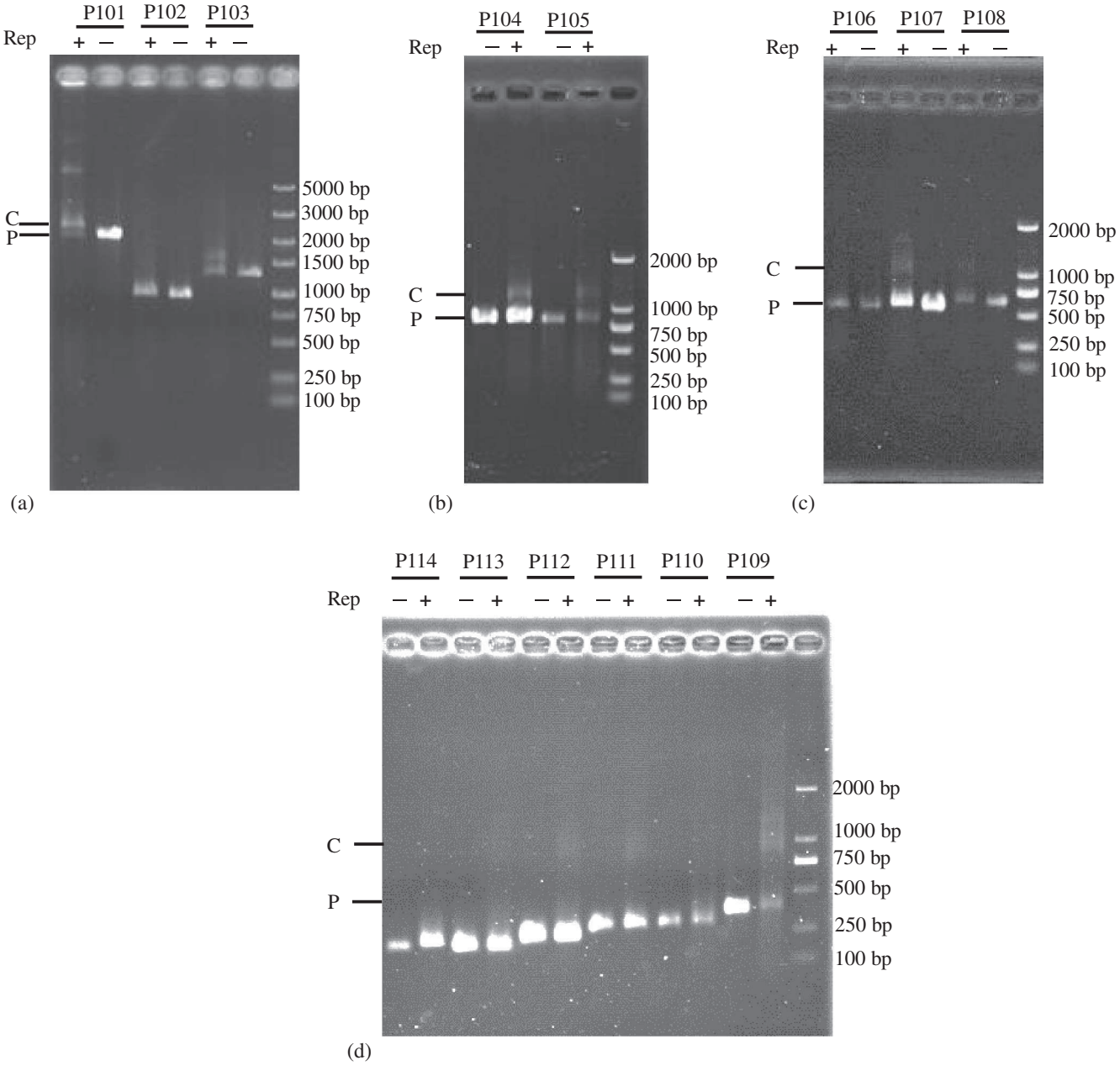

Figure 6. Identification of pG6303 Rep binding region. (a) P101, 4356-6160 bp; P102, 4356-5129 bp; P103, 5065-6160 bp; (b) P104, 5332-6160 bp; P105, 5065-5945 bp; (c) P106, 5065-5672 bp; P107, 5582-6160 bp; P108, 5332-5945 bp; (d) P109, 5582-5945 bp; P110, 5664-5945 bp; P111, 5582-5856 bp; P112, 5582-5793 bp; P113, 5776-5945 bp; P114, 5664-5856 bp. The positions of free PCR products (p) and Rep-DNA complex (c) are shown on the left side of the gels.

binds its OriV specifically (Wang *et al.* 1993). Theta plasmids need Rep proteins, OriV and host-encoded DNA polymerase I to trigger replication (Alpert *et al.* 2003). Plasmid pSK41 that replicates via theta mode has been studied in considerable detail and its Rep protein binds its OriV specifically too (Liu *et al.* 2012). However, the above results of EMSA experiments of pG6303 showed that its Rep protein cannot bind its OriV (4356–5129 bp) but can bind the 363 bp (5582–5945 bp) which contains two IRs in pG6303, while this 363 bp cannot support pG6303 replication in electroporation experiments. Moreover, in electroporation experiments, plasmid pUC63037 which contains the putative OriV of plasmid pG6303 can replicate in *L. plantarum* G63, and this result is contrary to the EMSA experiments. This phenomenon is different from RCR and theta replication. Based on these results, we speculate that the mode of replication of pG6303 may differ from RCR replication and theta replication and the binding of Rep from plasmid pG6303 is not necessary from the replication of plasmid pG6303. Further studies are necessary for a better understanding of its replication mechanism. Such studies will provide insight regarding the novel plasmid replication mode in nature.

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