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Detection and characterization of a theta-replicating plasmid pLP60 from *Lactobacillus plantarum* PC518 by inverse PCR

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ABSTRACT

Plasmid DNA of *Lactobacillus plantarum* PC518 was isolated by an improved method which contained a washing step for removing lysozyme. Three plasmid DNA libraries were constructed. A pair of outward primers was designed at both ends of the novel plasmid fragment obtained from plasmid DNA libraries, and the remainder of the circle plasmid was amplified by inverse PCR (iPCR). The whole sequence of plasmid was analyzed by the basic local alignment search tool, Tandem Repeats Finder, DNAMAN V6.0, DNASTAR and MEGA X software. The copy number was measured using quantitative real-time PCR. Plasmid extract showed 7 bands on agarose gel, indicating that *L. plantarum* PC518 contains multiple plasmids. The complete sequence of plasmid pLP60 was obtained by plasmid DNA libraries and iPCR. pLP60 is 6006 bp in length with a G + C content of 41.19 %, which encodes 8 open reading frames (ORFs). The *ori* site like theta-type could be located upstream of *repB*, which contains a short tandem repeats (sTR) and a long tandem repeats (lTR). RepB of pLP60 only had low similarity with Rep protein of known theta-type plasmids, but phylogenetic tree analysis showed that plasmids whose Rep proteins are similar to pLP60 have lTR at *ori*, and the conservativeness of lTR is consistent with similarity of Rep proteins, suggesting that RepB of pLP60 is a theta-replicating protein. So pLP60 was classified as class A of theta replication. The copy number of pLP60 was measured as 5 copies per cell by qPCR.

1. Introduction

Lactobacillus plantarum was recognized as a safe and multifunctional lactic acid bacterium [1], which was used for dairy products, meat and many vegetable fermentations. Some strains of *L. plantarum* harbored multiple plasmids, most of which remained cryptic [2]. In genome database of NCBI (national center for biotechnology information), 225 plasmids of *L. plantarum* had been annotated, the size of which vary from 0.8 to 127 kb (https://www.ncbi.nlm.nih.gov/genome/plasmids/1108?). Recently, novel plasmids of *L. plantarum* had been reported yet including pM411 [3], pLQ801 [4], pPLP-1 [5], and so on.

If there was a more effective way to analyze low-copy plasmids, more plasmids of *L. plantarum* may be reported. Inverse PCR (iPCR) [6], a method for amplifying unknown flanking sequences of known sequences, could be used to analyze plasmids which are hard to isolate and purify. Using iPCR, the full sequence of a plasmid could be determined based on just a partial known sequence, instead of purification of the whole plasmid DNA. The complete sequence of pCJ01 from *Campylobacter jejuni* was obtained by iPCR [7].

Circular plasmid had three modes of DNA replication, namely rolling circle (RC), theta type and strand displacement. The origin region (*ori*) of strand displacement replication always contains tandem repeats and inverted repeats, and requires three plasmid-encoded proteins. The *ori* of the RC-replication always contains a highly conserved *nic* region by which RC-replicating plasmids can be defined as a particular family [8]. But the *ori* of theta replication do not necessarily contain repetitive or conserved sequences [9]. Three classes of theta replication plasmids are reported, in which only class A plasmids carry a replication gene (*rep*) and an *ori* region with tandem repeats [10]. The *oris* of many theta-type plasmids extracted from gram-positive bacteria contain short tandem repeats (sTR) at A + T region and long tandem repeats (lTR) at the binding site of Rep, for example, PUCL22 [11], PUCL287 [12], pLME300 [13], pPLA4 [14] and pREN [15].

L. plantarum PC518 harbored multiple plasmids, from which plasmid pLP18 with a size of 1.8 kb had been sequenced and published previously by ourselves [16]. In the study, another new plasmid was also obtained from the strain by iPCR. The sequence analysis indicated it belonged to the theta-replicating plasmid. Through qPCR, the copy number of pLP60

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was measured as 5 copies per cell.

2. Materials and methods

2.1. Reagents and strains

L. plantarum PC518 was cultured stationarily in MRS broth at 37 °C, which was isolated from samples of Sichuan pickle made in China. *Escherichia coli* DH5**α** (Invitrogen) was cultured in LB medium at 37 °C pET-22b (+) (Invitrogen) was used as a sequencing vector. TaKaRa LA Taq (to amplify long chain DNA) and DNA Ligase were from TaKaRa Company (Dalian, China), and restriction endonuclease was purchased from ThermoFisher Scientific (Thermo scientific, USA).

2.2. Plasmid extraction

Pet22b (+) and recombinant pet22b (+) were extracted from *E. coli* DH5**α** by the eZNA plasmid mini kit (Omega Bio-Tek, USA), which method followed the manufacturer's manual. The cell of *L. plantarum* PC518 was treated by 100 mg/ml lysozyme for 10 min at 37 °C; then the cell was washed twice using 5 % glucose solution for removing lysozyme; finally, plasmid DNA was extracted using the eZNA plasmid mini kit.

2.3. Construction of plasmid DNA library

Among restriction endonucleases that cannot digest pLP18, *Hind* III, *Nco* I, and *Nde* I were chosen for the construction of plasmid DNA library of *L. plantarum* PC518 by single enzyme digestion. Plasmid DNA of *L. plantarum* PC518 and pET-22b (+) were digested with chosen restriction endonucleases respectively. After ligation reaction, transfer the mixture into *E. coli* DH5α using electrotransformation. The method of endonuclease digestions and ligations followed the supplier's manual. Finally, three plasmid DNA libraries were constructed. The recombinant plasmid in the plasmid DNA libraries were extracted and sequenced by Qingke Company (Chengdu, China). The fragments of plasmid DNA from libraries were aligned with the GenBank database by the basic local alignment search tool (BLAST). Only novel fragments were focused.

2.4. Inverse PCR

If a novel fragment was obtained from plasmid DNA libraries, a pair of outward primers will be designed at both ends of the novel fragment by Primer Premier 5 software (Premier Biosoft, Palo Alto, CA), and the remainder of the circle plasmid could be amplified by iPCR using plasmid extract from *L. plantarum* PC518 as template. The length of the amplified fragment was unknown, so the Taq polymerase for amplifying long chain DNA should be used. In order to well splice the template and the amplified fragment, two suitable overlapping areas should be set up.

2.5. Plasmid DNA sequence analysis

Sequence alignments were performed using BLAST at NCBI website (http://www.blast.ncbi.nlm.nih.gov/). Tandem repeats were searched using Tandem Repeats Finder at website (http://tandem.bu.edu/trf/trf.advanced.submit.html) and alignment parameters were set to 2, 3 and 5. DNAMAN V6.0 software was used to carry out multiple sequence alignments, locate the sites of restriction endonucleases, and detect direct or inverted repeats. The DNASTAR software package was utilized to predict open reading frames (ORFs). Phylogenetic trees were reconstructed using MEGA X software (version 10.0.4).

2.6. Determination of copy number

The copy number of pLP60 was measured using quantitative real-time PCR (qPCR). Total DNA was extracted from *L. plantarum* PC518 using the method reported by te Riele [17]. Amplification and detection were performed using a Bio-Rad iQ5 real-time PCR machine and a HotStart PCR Master mix (Bio Basic Inc., Ontario, Canada). Two pairs of PCR primers were designed by the Premier 5 software. A 150-bp fragment of pLP60 was amplified using primers pq15-F (5′-CCCCATAACACCCTACCT-3′) and pq15-R (5′-AAGACATTCTATCCCGAGT-3′), and a 170-bp fragment of pLP60 was amplified using primers pq17-F (5′-CTAAAGCGTCAACCGTAT-3′) and pq17-R (5′-TTCGTTCCCTTCGAGATG-3′). The gene of L-lactate dehydrogenase (*ldhL*) was used as the reference gene, and a 152-bp fragment of the

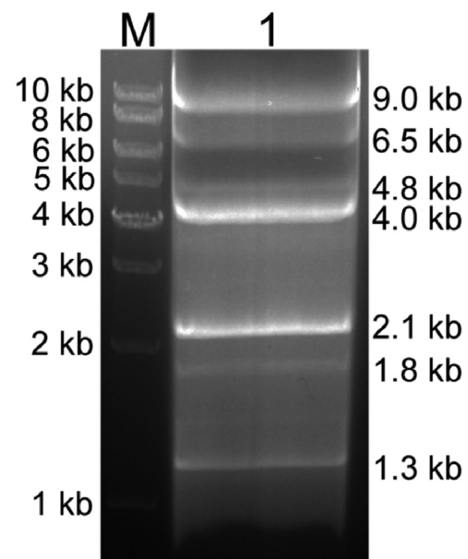

Fig. 1. Agarose gel electrophoretogram of plasmid DNA of *L. plantarum* PC518. Plasmid DNA of *L. plantarum* PC518 was extracted by an improved method which contained a washing step for removing lysozyme and used the Omega plasmid mini kit. The concentration of agarose gel was 1 % (w/v). Seven bands appeared on the gel after electrophoresed. Lane M: marker; Lane 1: plasmid DNA of *L. plantarum* PC518.

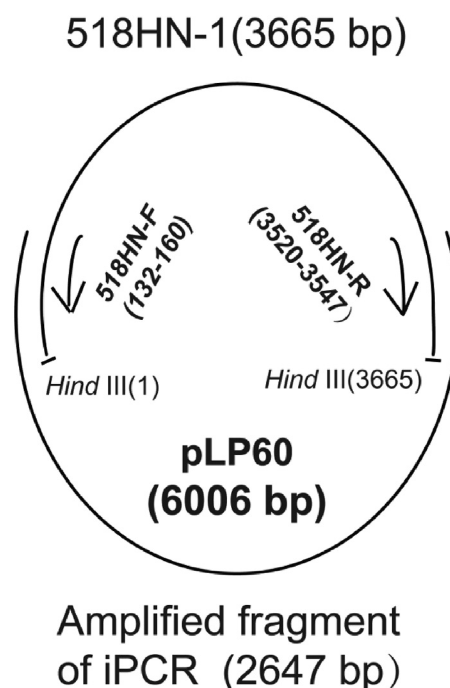

Fig. 2. The assembly diagram of plasmid pLP60. The lengths of two overlaping regions were 160 bp and 146 bp respectively.

ldhL gene was amplified [16]. The copy number of pLP60 was calculated using $N_{relatives} = (1 + E)^{-\Delta C}T$ [18]. The total DNA of *L. plantarum* PC518 was diluted continuously and the 10-fold dilution was utilized to construct standard working curves of *ldhL*, pq15 and pq17. All qPCRs were conducted three times, and the average revalues were used to calculate copy number.

2.7. Accession number of pLP60

The complete sequence of pLP60 was submitted to GenBank data base and the accession number is MH544243.

3. Results and discussion

3.1. Plasmid DNA of L. plantarum PC518

Seven bands appeared on gel when plasmid extract was analyzed by agarose gel electrophoresis (Fig. 1), indicating that *L. plantarum* PC518 contains multiple plasmids. Among which, plasmid pLP18 with a size of 1.8 kb had been sequenced and published previously by ourselves [16]. Compared with the past report, the number of bands on gel increased from four to seven because the method of plasmid extraction had been improved. In the improved method of plasmid extraction, the washing step for removing lysozyme was added.

3.2. Detection of plasmid pLP60

30 plasmid fragments of the plasmid DNA libraries were sequenced and aligned by BLAST, and one novel plasmid fragment with a size of 3665 bp was found, which was named 518HN-5. A pair of outward primers was designed at the sites with a distance of about 150 bp from both ends of 518HN-5, which were 518HN-F (5′- TCACTCA-GATTCATCAAAGTTTCGTGGTC -3′) and 518HN-R (5′-AGTGCTTCCT-TAGATGGTGATGGTAAAA-3′). Using iPCR, the amplified fragment with a length of 2647 bp was obtained. The lengths of two overlaping regions between fragment 518HN-5 and the amplified fragment were 160 bp and 146 bp respectively. Finally, the complete sequence was accomplished with the assembly process using software DNAMAN V6.0, and the plasmid with a length of 6006 bp was named as pLP60 (Fig. 2).

3.3. Sequence analysis of plasmid pLP60

Plasmid pLP60 is 6006 bp in length with a G + C content of 41.19 %. It contained four sites of *Hind* III. The larger part of pLP60 between *Hind* III (1395) and *Hind* III (5054) was the fragment 518HN-5 and the smaller part between them was identified by iPCR. pLP60 was a novel plasmid until February 6, 2018, when plasmid pB21AG02 was uploaded (GenBank accession no. CP025733). pB21AG02 is a component of the genome of *L. plantarum* strain B21, which was sequenced by Illumna Hiseq 2000

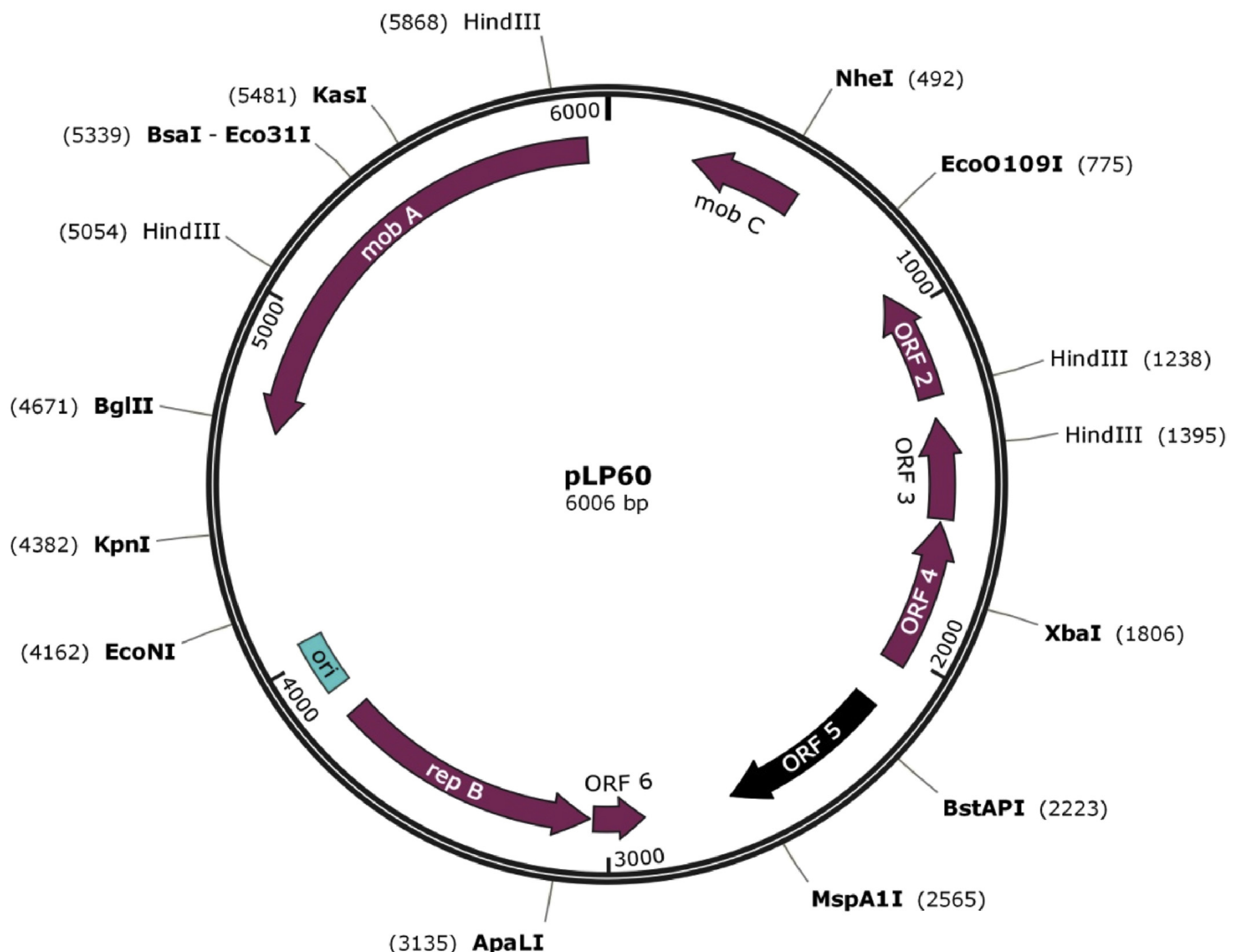

Fig. 3. Physical map of plasmid pLP60 from *L. plantarum* PC518. Eight ORFs were marked out using black or purple arrows. The *ori* site of replication and a number of restriction endonuclease sites were showed.

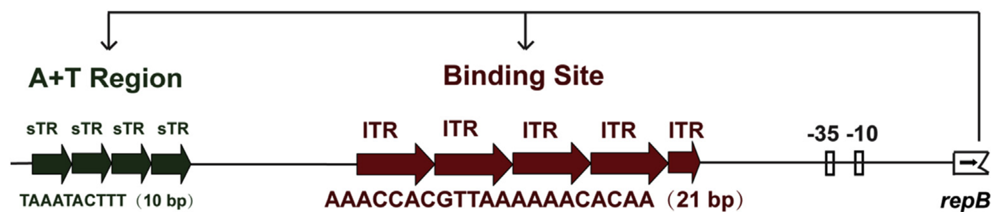

Fig. 4. The structural feature of the *ori* on pLP60. The short tandem repeats (sTR, 10 bp) and long tandem repeats (lTR, 21 bp) are indicated by green or red solid arrows respectively. RepB should recognize and bind lTR and then open double-strand DNA at sTR.

(https://www.ncbi.nlm.nih.gov/assembly/GCF_000931425.2/). pLP60 showed a 99 % identity with pB21AG02, but it still had very low sequence similarity with other plasmids, for example, the values of identity and query coverage of second-place plasmid (pLP12-4, GenBank accession no. CP018328) was 91 % and 37 % respectively.

It was predicted that pLP60 has eight ORFs containing more than 250 nucleotides (Fig. 3), and the eight ORFs shared 100 % similarity with that of pB21AG02. RepB of pLP60, a replication protein of 251 amino acids, had high similarity with Rep protein of several plasmids, including Rep B of pLP12-4 (96 %, GenBank accession no. APP13843 and APP13864), Rep_3 of pl12111-4 (87 %, ARN96593), Rep_3 of pL12108-8 (87 %, ARN94149), and RepB of pBGM48-5 (85 %, AUI80358). Mobilization protein C of 102 amino acids encoded by *mobC* had 97 % similarity with that of pLP2C (GenBank accession no. WP_103421027). Mobilization protein A of 433 amino acids encoded by *mobA* had a low similarity (61 % query coverage, 84 % identity) with that of plasmid pLP2C (GenBank accession no. WP_103421025). ORF1, ORF2, ORF3, ORF4 and ORF5 had no obvious similarity with all known proteins of the GenBank databases, so function of these proteins encoded by the ORFs cannot be deduced. The replicating origin (*ori*) of pLP60 located between *mobA* and *repB*.

3.4. Deduction of replication mechanism of pLP60

The *ori* containing an AT-rich region and a binding site could be located upstream of *repB*, where a sTR of 10-bp (TAAATACTTT) repeats 4.1 times at the AT-rich region, and a lTR of 21-bp (AAACCACGT-TAAAAAACACAA) repeats 4.4 times at the binding site (Fig. 4). In view of pLME300, pLP5401, pPLA4, pCD01 and pRCEID7.6 that had been classified as theta-replicating plasmids [13, 21, 14, 19, 20], this organization resembles the typical feature of class A theta replicons.

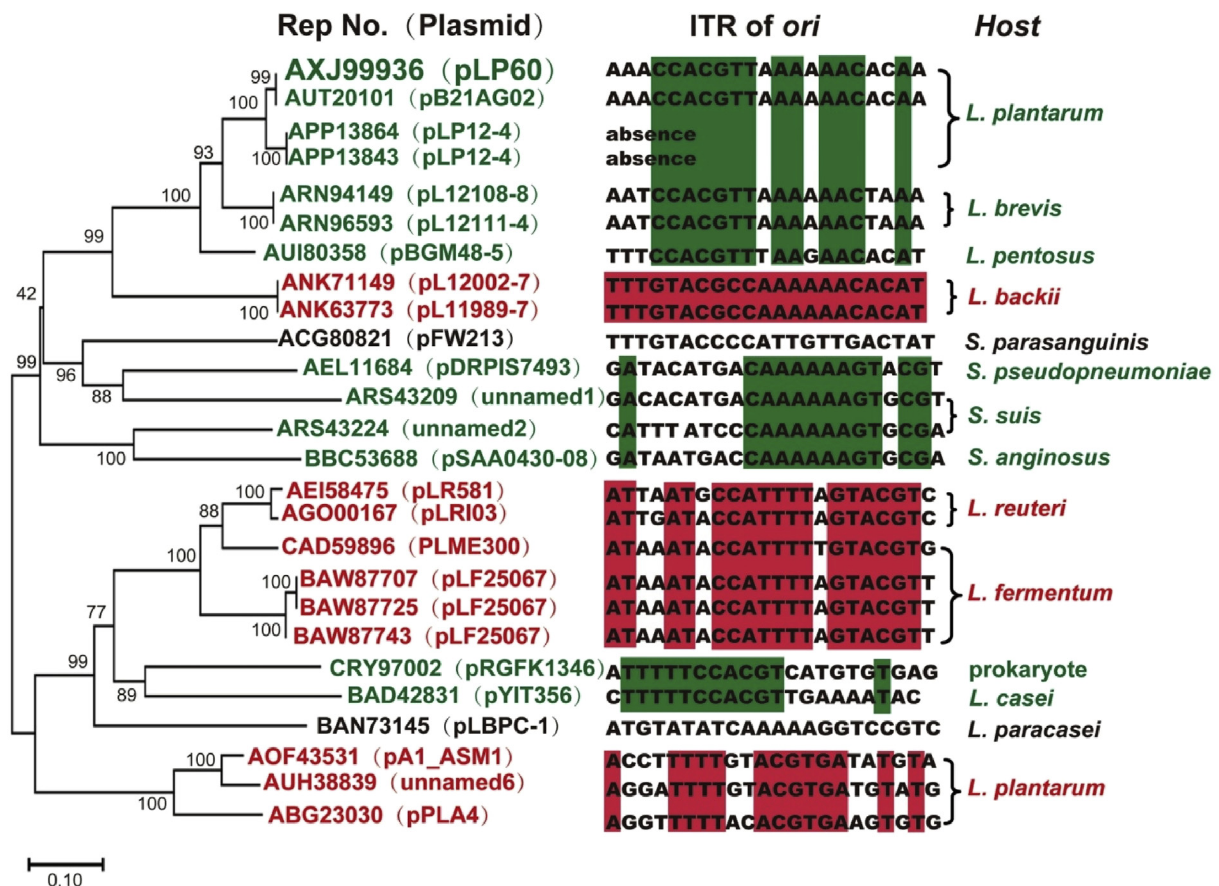

Fig. 5. Phylogenetic tree based on amino acid sequences of RepB of pLP60. The similarity rates of all Rep proteins in the evolutionary tree with RepB of pLP60 are greater than 45 %, and the coverage rates are more than 90 %. Red and green shadows mark conserved sequences of lTR (long tandem repeats). The phylogenetic tree was constructed using the Neighbor-Joining model. The percentages of the bootstrap consensus tree which was inferred from 1000 replicates were marked out next to the branches. Evolutionary analyses were conducted in MEGA X (version 10.0.4).

RepB of pLP60 only had low similarity with Rep protein of known theta-type plasmids. Among them, Rep protein of pPLA4 [14] and pLME300 [13] were most similar to RepB of pLP60 with the similarity of 52 % and 49 % respectively. To further analyze RepB of pLP60, the phylogenetic tree was constructed using the Neighbor-Joining model, which contained 25 Rep proteins of plasmids that values of query coverage and identity are more than 90 % and 45 % respectively (Fig. 5). Upstreams of the 25 *rep* genes, lTR was identified by Tandem Repeats Finder, except for two *rep* genes of pLP12-4. pLP12-4 contains three *rep* genes in which the *rep* gene (GenBank accession no. APP13853) with very low similarity to *repB* of pLP60 has lTR and sTR at the upstream. sTR could be located upstreams of 19 *rep* genes, but no sTR could be detect at the upstreams of the *rep* genes of pLP12-4, pl12111-4, pLRI03, pLR581and pRGFK1346, suggesting that lTR is a more conservative structural feature of *ori* than sTR. The length of lTR is relatively stable, either 21 bp or 22 bp, and the repetitions range from 3.4 to 6.6, which may control plasmid copy number [22]. But the sequences of lTRs are diverse. 24 lTRs on the evolutionary tree do not share a similar sequence, but the lTRs of similar Rep proteins have distinct conserved sequences, such as the base labeled with red or green shadows in Fig. 5. There have 13 conserved bases in the lTRs of pLP60, pB21AG02, pL12108-8, pL12111-4 and pBGM48-5 whose Rep proteins share >85 % similarity; Rep proteins of pDRPIS7493, unnamed1, unnamed2 and pSAA0430-08 have only more than 50 % similarity, but their lTRs still have 12 conserved bases. It suggested that classification of theta replication based on conservative sequence of lTR is more reliable than that based on similarity of Rep proteins. To reasonably deduction, all plasmids on the evolutionary tree should be replicated though theta-type except for pLP12-4. In addition, the plasmids in the evolutionary tree were isolated from 2 genera and 12 distinct species, suggesting that the host range of these theta -replicating plasmids might be narrow.

3.5. Copy number of pLP60

The copy number of pLP60 was measured by qPCR. The results showed that the standard working curves of *ldhL*, pq15 and pq17 were all linear ($R > 0.9990$) in the experimental range. The copy number of pLP60 was calculated to be 3.4 or 5.7 copies per chromosome equivalent according to the standard curves of *ldhL* and pq15 or *ldhL* and pq17, respectively. So the average copy number was about 5 copies per cell. The novel plasmid is a low-copy plasmid.

4. Conclusions

Plasmid pLP60 of *L. plantarum* PC518 was detected by plasmid DNA library, BLAST at NCBI website and iPCR, whose *ori* has the typical feature of theta replication. Rep protein of pLP60 has low similarity with known theta replicating plasmids, but phylogenetic tree analysis showed that plasmids which Rep proteins are similar to pLP60 have lTR at *ori*, and the conservativeness of lTR is consistent with similarity of Rep proteins, suggesting that RepB of pLP60 is a theta-replicating protein. So the plasmid was classified as class A of theta replication. The copy number of pLP60 was measured as 5 copies per cell by qPCR. Our method may be used to detect all multi-plasmid systems in bacteria. The classification of class A theta plasmid based on lTR of the *ori* should be more scientific than the traditional method by similarity of Rep protein.

Declarations

Author contribution statement

Fang Yao: Performed the experiments; Wrote the paper.

Qu Pan: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kang Cao: Analyzed and interpreted the data.

Xiaoyu Xu, Xin Du: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study (the complete sequence of pLP60) has been deposited at GenBank under the accession number MH544243.

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