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 (ITR). 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 ITR at ori, and the conservativeness of ITR 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, 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 ori of many theta-type plasmids extracted from gram-positive bacteria contain short tandem repeats (STR) at A + T region and long tandem repeats (ITR) 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...
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* DH5α (Invitrogen) was cultured in LB medium at 37 °C and 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* DH5α 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.ncbi.nlm.nih.gov/). Tandem repeats were searched using Tandem Repeats Finder website (http://tandem.bu.edu/trf/trfadvanced.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’- CCCATAACACCCGTACCT-3’) and pq15-R (5’- AAGACATTCTATCCCGAGT-3’), and a 170-bp fragment of pLP60 was amplified using primers pq17-F (5’-CTAAAGGTCGTAACCGTAT-3’) and pq17-R (5’-TTCTGGTCCCTTCGGAGATG-3’). The gene of L-lactate dehydrogenase (*ldhL*) was used as the reference gene, and a 152-bp fragment of the

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Fig. 1. Agarose gel electrophoresogram 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 overlapping regions were 160 bp and 146 bp respectively.
ldhl gene was amplified [16]. The copy number of pLP60 was calculated using 

\[ N_{\text{relatives}} = (1 + E)^{-\Delta C_T} \]  

[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- GATTCTAAAAGTTTGCGTGC-3') and 518HN-R (5'-AGTGCTCTC- TAGATGCGATGGTAAA-3'). Using iPCR, the amplified fragment with a length of 2647 bp was obtained. The lengths of two overlapping 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 Illumina Hiseq 2000.

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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.
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 RepB of pLP12-4 (96%, GenBank accession no. APP13843 and APP13864), Rep3 of pLP1211-4 (87%, ARN96593), Rep3 of pLP12108-8 (87%, ARN94149), and RepB of pBGM48-5 (85%, AU180358). 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 an itR of 21-bp (AAACCACGTTAACACAGAA) 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. 4. The structural feature of the ori on pLP60. The short tandem repeats (stR, 10 bp) and long tandem repeats (itR, 21 bp) are indicated by green or red solid arrows respectively. RepB should recognize and bind itR and then open double-strand DNA at stR.](https://www.ncbi.nlm.nih.gov/assembly/GCF_000931425.2/).

![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 itR (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).](https://www.ncbi.nlm.nih.gov/assembly/GCF_000931425.2/)
Repl of pLP60 only had low similarity with Rep protein of known theta-type plasmids. Among them, Rep protein of pLA4 [14] and pME300 [13] were most similar to Rep of pLP60 with the similarity of 52 % and 49 % respectively. To further analyze Repl 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). Upstream of the 25 rep genes, ITR 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. AP13853) with very low similarity to Repl of pLP60 has ITR and sTR at the upstream. sTR could be located upstream of 19 rep genes, but no sTR could be detected at the upstream of the rep genes of pLP12-4, pLP12111-4, pLR03, pLR581 and pRGFK1346, suggesting that ITR is a more conservative structural feature of ori than sTR. The length of ITR 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 ITRs are diverse. 24 ITRs on the evolutionary tree do not share a similar sequence, but the ITRs 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 ITRs of pLP60, pB21AG02, pL12108-8, pL12111-4 and pBGM48-5 whose Rep proteins share >85 % similarity; Rep proteins of pDPRPS7493, unnamed1, unnamed2 and pSAO430-8 have only more than 50 % similarity, but their ITRs still have 12 conserved bases. It suggested that classification of theta replication based on conservative sequence of ITR 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 > 0.9999) 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 PCS18 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 ITR at ori, and the conservativeness of ITR is consistent with similarity of Rep proteins, suggesting that RepB of pLP60 is a theta-replicating protein. So plasmid was classified as class A of theta replication. The classification of class A theta plasmid based on ITR 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.

References

[1] B. Klarin, G. Molin, B. Jeppsson, A. Larson, Use of the probiotic Lactobacillus plantarum 299 to reduce pathogenic bacteria in the oropharynx of intubated patients: a randomised controlled open pilot study, Crit. Care 12 (2008) R36.
[2] J.L. Ruiz-Barba, J.C. Piard, R. Jimenez-Diaz, Plasmid profiles and curing of plasmids in Lactobacillus plantarum strains isolated from green olive fermentations, J. Appl. Bacteriol. 71 (1991) 417–421.
[3] X. Ma, J. Li, Y. Xiong, Z. Zhai, F. Ren, Y. Hao, Characterization of a rolling-circle replication plasmid pML114 from Lactobacillus plantarum 1-3, Curr. Microbiol. 73 (2016) 820–826.
[4] N. Moriya, K. Nakano, A. Shiroma, M. Shinzato, N. Ashimine, M. Minami, H. Tamotsu, M. Shimoji, T. Nakanishi, S. Ohki, K. Teruya, K. Satou, T. Hirano, T. Hagi, M. Kobayashi, M. Nomura, H. Kimoto-Nira, K. Tajima, Y. Cai, C. Suzuki, Complete genome sequence of Lactobacillus plantarum strain LQ80, selected for preparation of fermented liquid feed for pigs, Genome Announc. 6 (2018) e00530–18.
[5] Y. Wang, N. Shang, Y. Qin, Y. Zhang, J. Zhang, P. Li, The complete genome sequence of Lactobacillus plantarum LPL-1a novel antibacterial probiotic producing class IIa bacteriocin, J. Biotechnol. 266 (2018) 84–88.
[6] H. Ochman, M.M. Medhora, G. Dan, D.L. Hartl, 27 – amplification of flanking sequences by inverse PCR, PCR Protocols (1990) 219–227.
[7] N. Luo, Q. Zhang, Moleculare characterization of a cryptic plasmid from Campylobacter jejuni, Plasmid 45 (2001) 127–133.
[8] S.A. Khan, Plasmid rolling-circle replication: highlights of two decades of research, Plasmid 53 (2005) 126–136.
[9] G.D. Solar, R. Giráldez, M. Espinosa, R. Díazorejas, Replication and control of circular bacterial plasmids, Microbiol. Mol. Biol. Rev. 62 (1998) 434–464.
[10] C. Bruand, E. Le Chatelier, S.D. Ehrlich, L. Janniere, A fourth class of theta-replicating plasmids: the pAM beta 1 family from gram-positive bacteria, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 11668–11672.
[11] J. Frere, M. Novel, G. Novel, Molecular analysis of the Lactococcus lactis subsp. lactis CNC2270 bidirectional theta-replicating lactose plasmid pCL22, Mol. Microbiol. 10 (1995) 1113–1124.
[12] A. Benachour, J. Frère, S. Fisahn, G. Novel, Y. Aufray, Molecular analysis of the replication region of the theta-replicating plasmid pSU287 from Tetragnococcus (Pedicoccus) ATCC23215, Mol. Gen. Genet. 255 (1997) 504–512.
[13] K.V. Géller, M. Roth, L. Meile, M. Treuber, Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pME500 from Lactobacillus fermentum ROTT1, Plasmid 50 (2003) 190–201.
[14] C.A. Van Reemen, W.H. Van Zyl, J.M. Dick, Expression of the immunity protein of proteinacin 423, produced by Lactobacillus plantarum 423, and analysis of the plasmid encoding the bacteriocin, Appl. Environ. Microbiol. 72 (2006) 7644–7651.
[15] I.A. Astero, K. Popadimitriou, E. Boutou, B. Pot, C.E. Vergias, E. Tsalakidou, Comparative and evolutionary analysis of plasmid pREN isolated from Lactobacillus ruminis, a novel member of the theta-replicating pCL287 family, FEMS Microbiol. Lett. 318 (2011) 18–26.
[16] Q. Pan, L. Zhang, J. Li, T. Chen, W. Chen, G. Wang, Characterization of pLP18, a novel cryptic plasmid of Lactobacillus plantarum PCS18 isolated from Chinese pickle, Plasmid 65 (2011) 204–209.
[17] H. te Riele, B. Michel, S.D. Ehrlich, Single-stranded plasmid DNA in Bacteriophages and Siphophlococcus aureus, Proc. Natl. Acad. Sci. U.S.A. 85 (1986) 2541–2545.
[18] C.J. Lee, D.S. Ow, S.K. Oh, Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria, J. Microbiol. Methods 65 (2006) 258–267.
[19] C. Desmond, R.P. Ross, G. Fitzgerald, C. Stanton, Sequence analysis of the plasmid genome of the probiotic strain Lactobacillus paracasei NFB138 which includes the plasmids pCDOI and pCD02, Plasmid 54 (2005) 160–175.
[20] N. Suebwongsa, V. Lulitanond, B. Mayo, P. Yotpanya, M. Panya, Development of an Erichschia coli-Lactobacillus casei shuttle vector for heterologous protein expression in Lactobacillus casei, SpringerPlus 5 (2016) 169–179.
[21] X. Zhu, Y. Zhao, C. Zhang, L. Shen, H. Jiang, Q. Gu, Characterization of three new plasmids from Lactobacillus paracasei 54, Turkish J. Biochem. 40 (2015) 265–270.
[22] K. Nordstrom, Control of plasmid replication–how do DNA iterons set the replication frequency? Cell 63 (1990) 1121–1124.