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Characterization of Four Novel Plasmids from *Lactobacillus plantarum* BM4

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Abstract

Background: *Lactobacillus plantarum* is a widespread probiotic bacterium. Many plasmids from *L. plantarum* have been identified to encode some important phenotypic traits including carbohydrate metabolism, bacteriocin synthesis, and exopolysaccharide production.

Objectives: The aim of this study was to identify and characterize the native plasmids from *L. plantarum* BM4.

Methods: *Lactobacillus plantarum* BM4 was isolated from fermented meat in Guangxi Province, China, and characterized by 16S rRNA sequence. Four plasmids were isolated from *L. plantarum* BM4, sequenced, and characterized by the bioinformatics method. Moreover, the relative copy numbers of these plasmids were estimated using the droplet digital PCR method.

Results: Four plasmids, designated as pBM1, pBM2, pBM3, and pBM4, were isolated from *L. plantarum* BM4. By nucleotide sequencing, pBM1, pBM2, pBM3, and pBM4 were characterized as having sizes of 6069 bp, 7042 bp, 8131 bp, and 8892 bp, and G+C contents of 37.5%, 36.7%, 36.4%, and 34.5%, respectively. Nucleotide sequence analysis revealed 8, 10, 10, and 10 putative open reading frames (ORFs) for pBM1, pBM2, pBM3, and pBM4 plasmids, respectively. Based on sequence alignment, only pBM2 contained replication protein RepB and rep3, which contained a putative repeat origin of replication segment, indicating that the pBM2 belongs to the pUCL287 subfamily of theta-type replicons. Finally, the relative copy numbers of pBM1-4 were estimated to be 82, 24, 34, and 16, copies, respectively.

Conclusions: Four novel plasmids were isolated from *L. plantarum* BM4 and characterized. These backbones can potentially be developed for use as a cloning or expressing vectors in biotechnology applications.

Keywords: Plasmid, Replication, *Lactobacillus plantarum*

1. Background

Lactobacillus plantarum is widely used in starter cultures in industrial food fermentation and it plays a beneficial role in health as a probiotic bacterium (1, 2). *Lactobacillus plantarum* species often harbor one or more natural plasmids (3). Many important properties of *L. plantarum*, such as resistance to phages, lactose catabolism, bacteriocins, and exopolysaccharides, are encoded by their plasmids (4). With the development of biology and genetics, characterization of the potential valuable tools of *L. plantarum* plasmids has become a hot area of research in recent years.

To date, many plasmids of *L. plantarum* have been sequenced, such as pLP9000 (9.3 kb) (5), pPB1 (2.9 kb) (6), and pLKS (2.0 kb) (7), and the number of sequenced plasmids have been steadily increasing. At least three different mechanisms of plasmid replication have been recognized, namely theta type, strand displacement, and rolling circle mode of replication (RCR), among which RCR is widespread in bacterial plasmids (8). Bacterial plasmids usually initiate replication by a plasmid-encoded protein, generically termed Rep (9).

In general, initiation of RCR needs recognition of the plasmid dso by the cognate Rep protein, involving the synthesis of single-strand DNA (ssDNA) intermediates (10). Based on sequence similarity in the Rep protein and double-stranded origin (dso), RCR plasmids can be grouped into several families, e.g., pT181, pE194/pLS1, pC194/pUB110, pSN2, and pMV158 (11). The theta type plasmids contain the rep gene and a replicon that consists of an origin of replication (ori) and an AT-rich region that primarily involves repeats as well as a binding site (12). Theta replicons comprise three key components, which are plasmid-encoded initiator Rep proteins, origins of replication, and host-encoded DNA polymerase I for nascent strand DNA synthesis (13). The ori of these lactococcal replicons consists of a set of short repeats (10 or 11 bp) and it is finished by large repeats (20 or 22 bp) adjacent to the promoter of the initiator gene (14). In addition, at least five

classes of theta replicons have been identified according to the mechanism of initiation of DNA replication (15, 16).

2. Objectives

In this paper, four novel plasmids named pBM1, pBM1, pBM1, and pBM4 were isolated from *L. plantarum* BM4. The nucleotides of the four plasmids were sequenced and characterized, and the copy numbers of the four plasmids in the cell were determined by Droplet Digital PCR.

3. Methods

3.1. Bacterial Strains, Plasmids and Growth Conditions

The *L. plantarum* BM4 used in this study was isolated from fermented meat in Guangxi Province, China. *Lactobacillus plantarum* BM4 was cultured on Man-Rogosa-Sharpe (MRS) medium (LuQiao, China) at 37°C under anaerobic conditions. *Escherichia coli* DH5a was used as a cloning host and cultured in Luria-Bertani (LB) medium (AoBoXing, China) with vigorous shaking at 37°C. Plasmid pBluescript II SK (+) (Stratagene, USA) was used as a subcloning vector for sequencing. When needed, ampicillin (at a final concentration of 100 μg/mL) was added to the medium.

3.2. 16S rRNA analysis of L. plantarum BM4

The genome DNA was isolated from *L. plantarum* BM4 using the TIANamp Bacteria DNA Kit (Tiangen, China) according to the manufacturer's instruction. The 16S rRNA gene sequences were amplified from *L. plantarum* BM4 genomic DNA using PCR technique (Bio-Rad, USA) with universal primers 27F and 1492R (Table 1). The amplified fragment was subjected to agarose gel electrophoresis (1% w/v), and sequenced (Sangon Biotech, China). The 16S rDNA gene sequences of *Lactobacillus* were collected from the Genbank database. MEGA 4.0 was used to perform multiple sequence alignments using the ClustalW method (17).

3.3. Plasmid Sequencing and Analysis

The plasmids were isolated from *L. plantarum* BM4 by the alkaline lysis method with a slight modification (18). Briefly, cells were harvested after overnight culture by centrifugation at 10,000 g for 5 minutes, and then washed in TES buffer (50 mM Tris-Cl, 30 mM EDTA, 25% Sucrose, pH 8.0). Lysozyme was subsequently added at the final concentration of 50 $mg.mL^{-1}$, and the suspension was incubated at 37°C for 1 hour. Plasmid DNA was isolated using a TIANprep Mini Plasmid Kit (Tiangen, China). The extracted total plasmid DNA was subjected to the 1% (w/v) agarose electrophoresis analysis.

Plasmid DNA isolated from *L. plantarum* BM4 and pBluescript II SK (+) was digested by KpnI Restriction endonuclease (RE) according to the supplier's instructions (Takara, China). The RE-digested plasmids were subsequently cloned into the pBluescript II SK (+) vector and transformed into *E. coli* DH5a. Positive clone was selected by Ampicillin agar (100 μg/mL). Plasmid DNA from positive *E. coli* was isolated following the TIANprep Mini Plasmid Kit according to the manufacturer's instruction (Tiangen, China). The inserted fragment was sequenced with T7 and T3 promoter primers using Applied Biosystems Automated Sequencer (Sangon Biotech, China), and the complete nucleotide sequence was obtained through primer walking method.

Open reading frames (ORF) were predicted by the ORF finder program at the national center for biotechnology information (NCBI) site and FGENESB program at the Softberry site (http://linux1.softberry.com/berry.phtml). Alignments of conserved protein domains were retrieved from the conserved domain database (CDD) at the NCBI (19). The DNASTAR software package was employed to detect direct and inverted repeats. The promoter was predicted at the Softberry site (http://linux1.softberry.com/berry.phtml).

3.4. Copy Number Determination by Digital PCR

The L-lactate dehydrogenase gene (ldhL1) (Genbank: NC_004567.2) in *L. plantarum* WCFS1, which was identified as a chromosomally encoded single-copy gene, was used as the reference gene. PCR primers used in digital PCR were shown in Table 1. A single ddPCR reaction volume of 20 μL contained 10 μL 2x ddPCR supermix (Bio-Rad, USA), 2 μL primers (final concentration of 10 μM), 2 μL DNA template, and 6 μL ddH_2O. Reaction mixtures were briefly mixed by vortexing while avoiding the formation of bubbles, microcentrifuged for 20 sec, and then kept on ice until droplet generation. Samples were converted into droplets with the QX100 Droplet Digital PCR (ddPCR) system (Bio-Rad, USA) according to the manufacturer's instruction. Next, the droplets were transferred from the droplet wells in the DG8 cartridges (Bio-Rad, USA) to a 96-well PCR plate and sealed for 5 seconds with a heat sealer. After that, amplification was performed in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under the following cycling conditions: 95°C for 6 minutes followed by 40 cycles of 95°C for 15 seconds and 52°C for 45 seconds. After PCR, the plate was loaded onto the QX100 Droplet Digital reader (Bio-Rad, USA), which automatically reads the droplets from each well of the plate.

3.5. Nucleotide Sequence Accession Number

Lactobacillus plantarum BM4 16S ribosomal DNA gene, partial sequence has been deposited in GenBank under

Table 1. Primers Used in This Study

Target	Sequence (5'-3')	Amplification Size, bp
16S rRNA	27F: AGAGTTTGATC-CTGGCTCAG	1465
	1492R: TACGGCTAC-CTTGTTACGACTT	
ldhL1	ldh-F: CACCGTCTTC-TAACTTGGCT	152
	ldh-R: TCCTCGTTC-CGTTGATGC	
pMB1	pBM1-F: TAGCAC-GATTTTGACCAG	116
	pBM1-R: CACCAAGC-GAAACTAACG	
pMB2	pBM2-F: TCTTATTA-GATGGGCTATTTG	190
	pBM2-R: GGATTATCA-GAGGCAAGGT	
pMB3	pBM3-F: CAGC-CGTTGACCTATTGC	130
	pBM3-R: TTCGCTTGGT-GTTTTGTTT	
pMB4	pBM4-F: TGCCAACGAG-GAAAATCA	111
	pBM4-R: AATCAACCA-GACCACGGA	

Accession No. KP976095.1. The complete nucleotide sequences of pBM1-4 have been deposited in GenBank (Accession No: KT149387, KT149388, KT149389, KT149390).

3.6. Statistical analysis

Date analysis was performed using Quantasoft software (Bio-Rad, USA). The plasmid relative copy number to the chromosome was calculated based on the equation: PCN (plasmid copy number) = Np/Nc, where Np is the copy number per μL of the plasmid target gene, and NC is the copy number per μL of the control (chromosomal target gene) with the same template. The experiments were performed in triplicate, and the average values were recorded.

4. Results

4.1. Isolation and Identification of L. plantarum BM4

Lactobacillus strain BM4 was originally isolated from fermented meat. The 16S rDNA gene sequence analysis indicated that strain BM4 shows 99% homology with *L. plantarum* strains (Figure 1). Consequently, the *Lactobacillus* strain BM4 belonged to the *L. plantarum* species and therefore, it was designated as *L. plantarum* BM4.

4.2. Sequence Analysis of Plasmid pBM1-4

*Lactobacillus plantarum*BM4 harbored four plasmids, designated as pBM1, pBM2, pBM3, and pBM4, respectively. To sequence the plasmids, the four plasmids were digested with KpnI and then cloned into the vector pBluescript II SK (+). The sequence analysis results revealed that pBM1, pBM2, pBM3, and pBM4 were 6069 bp, 7042 bp, 8131 bp, and 8891 bp in length and possessed average G + C contents of 37.5%, 36.7%, 36.4%, and 34.5%, respectively. All open reading frames larger than 40 amino acids and their potential functions have been predicted at NCBI in the GenBank database. Physical maps of plasmids pBM1-4 are shown in Figure 2. We predicted 8 ORFs on pBM1, and 10 ORFs on pBM2, pBM3, and pBM4.

4.3. Rep Proteins in Plasmid pBM2

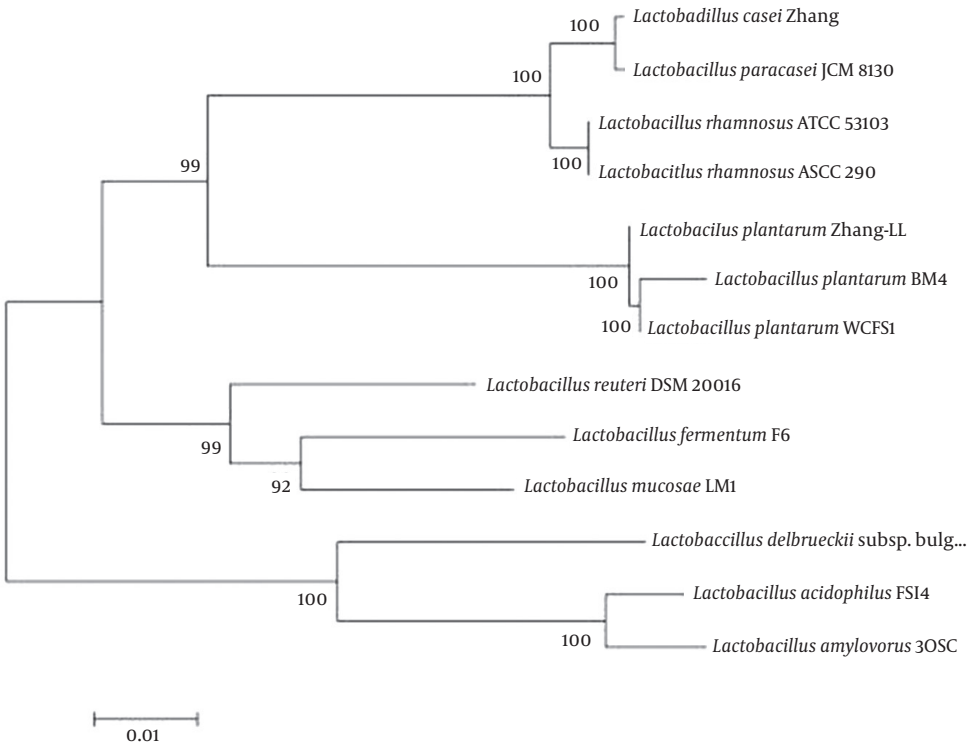
According to the sequence analysis, among the four plasmids isolated from *L. plantarum* BM4, only pBM2 encoded a plasmid probable replication protein (repB) and an initiator replication family protein (repA), and the two genes overlapped in pBM2. Homology analysis results are shown in Figure 3. The repA protein (GenBank: ALO75838.1) of pMB2 shares 99% identity with the rep protein (GenBank: BAN08206.1) of plasmid pKB290-8, 80% identity with the repA protein (GenBank:CAA53278.1) of plasmid pUCL287, 86% identity with the repA protein (GenBank: NP_862285.1) of plasmid pMD5057, 78% identity with the repA protein (GenBank: NP_862269.1) of plasmid pRV500, 81% identity with the repB protein (GenBank: NP_857600.1) of plasmid pSMB74, and 78% identity with the initiation protein repA (GenBank: YP_002567764.1) of plasmid pRS5, all of which belong to the theta-replicating group.

In addition, supposed origins of replication (termed ori) are predicted upstream of repA (sites: 5749-6000) according to the structure feature, which consists of three direct 11-bp repeats (three times) and four 22-bp iterons, tandemly repeated (Figure 4). Moreover, Orf8 of pBM2 encoded a putative repB protein (GenBank: ALO75837.1) of 171 amino acids and shared 84% identity with the repB protein (GenBank: BAN08207.1) of plasmid pKB290-8 and 65% identity with the replication protein (GenBank: NP_857601.1) of plasmid pSMB74.

4.4. Other ORFS in Plasmid

All four plasmids contain a similar INT_C_like_3 Integrase protein of 195aa (orf5 in pBM1, orf1 in pBM2, orf3 in pBM3, and orf9 in pBM4), share over 90% identity with integrase of plasmids from *Lactobacillus pentosus* IG1 and *Lactobacillus pentosus* MP-10, and a transposase protein of 167aa (orf8 in pBM1, orf7 in pBM2, orf10 in pBM3, and orf4

Figure 1. A Phylogenetic Tree was Constructed with the MEGA Version 4 Program Using 16S rDNA Gene Sequences



Data for 16S rDNA phylogenetic analysis were obtained from the Genbank nucleotide sequence database for the following strains: *Lactobacillus casei* Zhang (GenBank: CP001084.2), *Lactobacillus paracasei* JCM8130 (GenBank: AP012541.1), *Lactobacillus rhamnosus* ATCC53103 (GenBank: FM179322.1), *Lactobacillus rhamnosus* ASCC 290 (GenBank: CP014645.1), *Lactobacillus plantarum* Zhang-LL (GenBank: CP011769.1), *Lactobacillus plantarum* WCFS1 (GenBank: AL935263.2), *Lactobacillus reuteri* DSM 20016 (GenBank: CP000705.1), *Lactobacillus fermentum* F-6 (GenBank: CP005958.1), *Lactobacillus mucosae* LM1 (GenBank: CP011013.1), *Lactobacillus delbrueckii* subsp. *Bulgaricus* ATCC 11842 (GenBank: CR954253.1), *Lactobacillus acidophilus* FSI4 #: Characterization of Four Novel Plasmids from... Revision 1 Journal: Jundishapur Journal o... Page 45 of 49 11 May 2017 13:18:08 (GenBank: CP010432.1), *Lactobacillus amylovorus* 30SC (GenBank: CP002559.1).

in pBM4), share over 98% identity with transposase of plasmids from *L. plantarum* subsp. plantarum P-8. The four integrase proteins of pBM1-4 belong to a superfamily of DNA breaking/re-joining enzymes and might play a role in plasmid multimer resolution. Orf3 and orf4 in pBM1, as well as orf4 and orf5 in pBM2, encode a toxin-antitoxin (TA) plasmid maintenance system. Orf3-encoding protein in pBM1 and orf5 encoding protein in pBM3 belong to plasmid stabilization system protein. Orf2-encoding protein in pBM2 belongs to antidote-toxin recognition MazE. MazE is the antidote to the toxin MazF of *E. coli*, which regulates the prokaryotic chromosomal addiction module. Orf3 in pBM2 encodes a PemK-like protein, which is an inhibitor for growing in *E. coli* known to bind to the promoter region of the Pem operon, auto-regulating synthesis. No obvious replication protein, double-strand origin, or single-strand origin was found in plasmids pBM1, pBM3, or pBM4. Therefore, our data do not support their replication mechanism. The detailed characteristics of pBM1-4 are shown in Table 2.

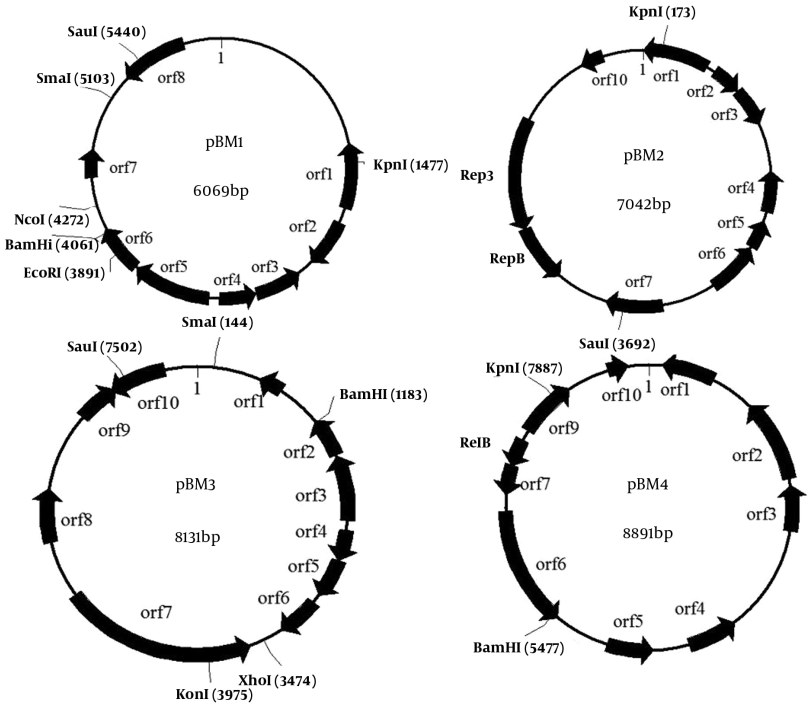
4.5. Relative Copy Number of pBM1-4

In this study, the relative copy numbers of pBM1, pBM2, pBM3, and pBM4 were determined by Droplet Digital PCR. Our results revealed that the relative copy numbers of pBM1, pBM2, pBM3, and pBM4 were approximately 82, 24, 34, and 16 copies per chromosome equivalent.

5. Discussion

In this work, four native plasmids were isolated from *L. plantarum* BM4. The sequence analysis results revealed that pBM1, pBM2, pBM3, and pBM4 were 6069 bp, 7042 bp, 8131 bp and 8891 bp in length, and we predicted 8 ORFs on pBM1, and 10 ORFs on pBM2, pBM3, and pBM4. According to our results, only pBM2 encoded an overlapping plasmid probable replication protein (repB) and an initiator

Figure 2. Physical Maps of Plasmid pBM1-4



The ORFs are indicated by closed arrows in their direction of synthesis. The repB and rep3 regions are indicated. Unique restriction enzyme sites are also shown.

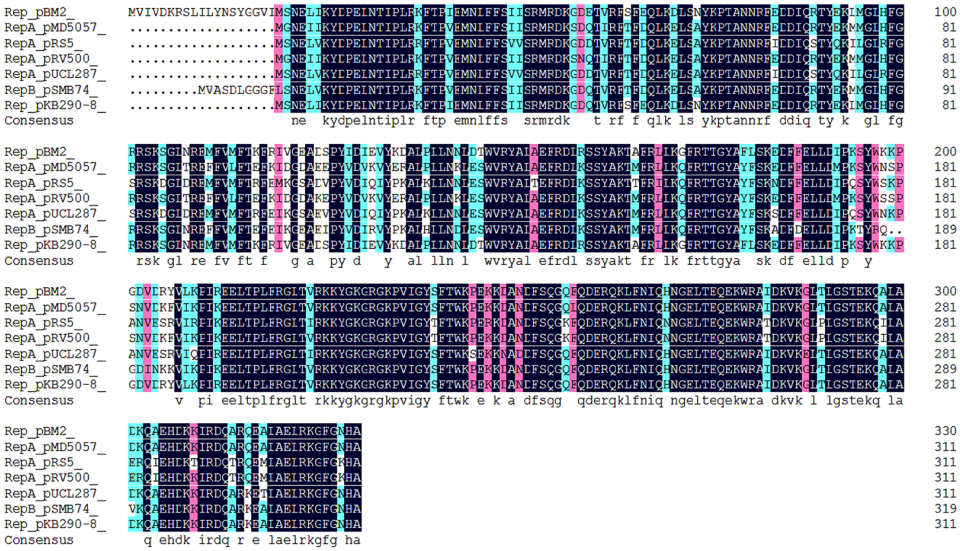
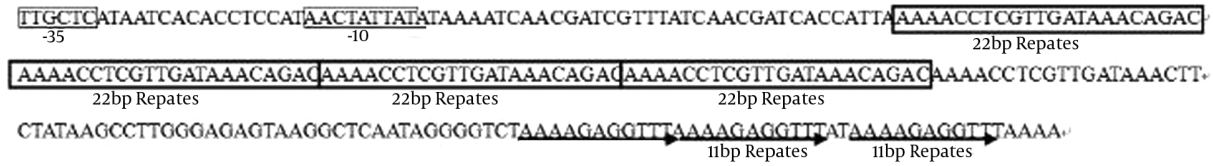


Figure 3. Amino Acid Sequence Alignment of the orf9-Coding Protein (RepA) of pBM2 with related plasmids RepA of PMD5057 (GenBank: AAN40880.1), RepA of pRS5 (GenBank: YP_002567764.1), RepA of pRV500 (GenBank: AAN61991.1), RepA of PUCL287 (GenBank: CAA53278.1), RepB of pSMB74 (GenBank: AAP55632.1).

replication family protein (repA). The repA protein (GenBank: ALO75838.1) of pMB2 shares 99% identity with the rep protein of plasmid pKB290-8 from *Lactobacillus brevis* KB290 (20), 80% identity with the repA protein of plas-

Figure 4. Structure of the Predicted Origin of Replication in Plasmid pBM2



The position is from 5749 to 6000. The predicted promoter (-35.-10) sequence is depicted by the black box, and the 11-bp repeat sites are labeled.

mid pUCL287 from *Pediococcus halophilus* ATCC33315 (21), 86% identity with the repA protein of plasmid pMD5057 from *Lactobacillus plantarum* 5057 (22), 78% identity with the repA protein of plasmid pRV500 from *L. sakei*, 81% identity with the repB protien of plasmid pSMB74 from *Pediococcus acidilactici* H (23), and 78% identity with the initiation protein repA of plasmid pRS5 from *P. pentosaceus* (24), all of which belong to the theta-replicating group.

Moreover, a typical ori was predicted upstream of repA (sites: 5749 - 6000), which consists of three direct 11-bp repeats (three times) and four 22-bp iterons, tandemly repeated (Figure 4). It has been reported that the 22-bp introns are important for pSB01 and the 11-bp repeats are necessary for pUCL287 replication (Benachour et al. 1997) (14, 25). Interestingly, the overlap of genes coding repA and repB was found in several theta-replicating plasmids, such as pKB290-8 from *Lactobacillus brevis* KB290, plasmid pSMB74 from *Pediococcus acidilactici* H, and plasmid pUCL287 from *Pediococcus halophilus* ATCC33315. It has been previously reported that RepB in pUCL287 is involved in plasmid stability and regulation of plasmid copy number, but is not essential for replication. In conclusion, these features suggest that pBM2 belongs to the group of pUCL287 theta-replicating plasmids (13, 26).

All four plasmids contain a similar INT_C_like_3 Integrase protein of 195aa, which has been previously reported that integrase in pSMB 74 is likely to be involved in recombination or integration (23). Tyrosine recombinase (integrase) belongs to a DNA breaking-rejoining enzyme superfamily. Many DNA breaking-rejoining enzymes also have N-terminal domains, which show little sequence or structure similarity (27). A toxin-antitoxin (TA) plasmid maintenance system was identified in pBM1 and pBM3, which belong to plasmid stabilization system protein in gram-negative and gram-positive bacteria, and may play a role in keeping low copy-number plasmids stable through neutralization toxin (2). The exact molecular function of these proteins is not known. This family also encompasses RelE/ParE, which seems to occur in pairs and to be organized as an operon (28).

Orf2-encoding protein in pBM2 belongs to antidote-toxin recognition MazE, which is the antidote to the toxin MazF of *E. coli*, and regulates the prokaryotic chromosomal addiction module. MazE-MazF is thought to play a role in programmed cell death when cells suffer nutrient deprivation, and MazE-MazF modules have been implicated in the bacteriostatic effects of other addiction modules (19). Orf3 in pBM2 encodes a PemK-like protein, which is an inhibitor for growing in *E. coli* known to bind to the promoter region of the Pem operon, auto-regulating synthesis. This Pfam family consists of the PemK protein in addition to ChpA, ChpB, and other PemK-like proteins (19).

Our data do not support the replication mechanism of plasmids pBM1, pBM3, or pBM4. However, it was reported that some plasmids without replication protein replicate via an RNA-based replication mechanism, such as pCD033 from *L. plantarum* 3NSH, and plasmid pColE1 (29-31). The principle of ddPCR involves subdividing a single PCR reaction mixture into many small partitions and each undergoing the PCR reaction separately (32). Several studies have reported using ddPCR to determine the relative copy numbers of plasmids (33, 34). According to our results, the relative copy numbers of pBM1, pBM2, pBM3, and pBM4 were calculated as 82, 24, 34, and 16 copies per chromosome equivalent using the ddPCR method. Our results suggest that pBM1-4 belong to the low copy plasmids.

6. Conclusion

In summary, we characterized four new plasmids, pBM1, pBM2, pBM3, and pBM4, isolated from *L. plantarum* BM4. By sequence analysis and comparison, we found that, only pBM2 contained replication protein RepB and rep3, which coding for a putative initiator protein contained a putative 11-and 22-bp repeat origin of replication segment, indicating the pBM2 belongs to the pUCL287 subfamily of theta-type replicons. Moreover, other proteins in pBM1-4 also involved module toxin, antitoxin, integrase, AbrB family transcriptional regulator, transposase, endonuclease, and translation repressor RelE. The relative copy numbers

of pBM1-4 were estimated to be 82, 24, 34, and 16 copies, respectively.

Footnotes

Authors' Contribution: Study concept and design: Yuanhong Xie; analysis and interpretation of data: Linxia Jie, Junchao Zhang, Junhua Jin; drafting of the manuscript: Linxia Jie, Hongxing Zhang; critical revision of the manuscript for important intellectual content: Yuanhong Xie, Hui Liu; statistical analysis: Linxia Jie, Junchao Zhang.

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References

1. Klarin B, Molin G, Jeppsson B, Larsson A. 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.* 2008;**12**(6):R136. doi: 10.1186/cc7109. [PubMed: 18990201].
2. Siezen RJ, Francke C, Renckens B, Boekhorst J, Wels M, Kleerebezem M, et al. Complete resequencing and reannotation of the Lactobacillus plantarum WCFS1 genome. *J Bacteriol.* 2012;**194**(1):195–6. doi: 10.1128/JB.06275-11. [PubMed: 22156394].
3. Fan J, Xi X, Huang Y, Cui Z. Isolation of a minireplicon of the plasmid pG6303 of Lactobacillus plantarum G63 and characterization of the plasmid-encoded Rep replication protein. *J Genet.* 2015;**94**(2):177–86. [PubMed: 26174665].
4. Zhou H, Hao Y, Xie Y, Yin S, Zhai Z, Han B. Characterization of a rolling-circle replication plasmid pXY3 from Lactobacillus plantarum XY3. *Plasmid.* 2010;**64**(1):36–40. doi: 10.1016/j.plasmid.2010.03.003. [PubMed: 20353802].
5. Daming R, Yinyu W, Zilai W, Jun C, Hekui L, Jingye Z. Complete DNA sequence and analysis of two cryptic plasmids isolated from Lactobacillus plantarum. *Plasmid.* 2003;**50**(1):70–3. [PubMed: 12826059].
6. de las Rivas B, Marcobal A, Muñoz R. Complete nucleotide sequence and structural organization of pPB1, a small Lactobacillus plantarum cryptic plasmid that originated by modular exchange. *Plasmid.* 2004;**52**(3):203–11. doi: 10.1016/j.plasmid.2004.09.001.
7. Eguchi T, Doi K, Nishiyama K, Ohmomo S, Ogata S. Characterization of a phage resistance plasmid, pLKS, of silage-making Lactobacillus plantarum NGRI0101. *Biosci Biotechnol Biochem.* 2000;**64**(4):751–6. doi: 10.1271/bbb.64.751. [PubMed: 10830488].
8. Ma X, Li J, Xiong Y, Zhai Z, Ren F, Hao Y. Characterization of a Rolling-Circle Replication Plasmid pM411 from Lactobacillus plantarum 1-3. *Curr Microbiol.* 2016;**73**(6):820–6. doi: 10.1007/s00284-016-1124-7. [PubMed: 27592105].
9. Ruiz-Maso JA, Lopez-Zumel C, Menendez M, Espinosa M, del Solar G. Structural features of the initiator of replication protein RepB encoded by the promiscuous plasmid pMV158. *Biochim Biophys Acta.* 2004;**1696**(1):113–9. [PubMed: 14726211].
10. Sanchez C, Hernandez de Rojas A, Martinez B, Arguelles ME, Suarez JE, Rodriguez A, et al. Nucleotide sequence and analysis of pBL1, a bacteriocin-producing plasmid from Lactococcus lactis IPLA 972. *Plasmid.* 2000;**44**(3):239–49. doi: 10.1006/plas.2000.1482. [PubMed: 11078650].
11. Khan SA. Plasmid rolling-circle replication: highlights of two decades of research. *Plasmid.* 2005;**53**(2):126–36. doi: 10.1016/j.plasmid.2004.12.008. [PubMed: 15737400].
12. van Belkum MJ, Stiles ME. Characterization of the theta-type plasmid pCD3.4 from Carnobacterium divergens, and modulation of its host range by RepA mutation. *Microbiology.* 2006;**152**(Pt 1):171–8. doi: 10.1099/mic.0.28294-0. [PubMed: 16385127].
13. Alpert CA, Crutz-Le Coq AM, Malleret C, Zagorec M. Characterization of a theta-type plasmid from Lactobacillus sakei: a potential basis for low-copy-number vectors in lactobacilli. *Appl Environ Microbiol.* 2003;**69**(9):5574–84. [PubMed: 12957947].
14. Benachour A, Frere J, Flahaut S, Novel G, Auffray Y. Molecular analysis of the replication region of the theta-replicating plasmid pUCL287 from Tetragenococcus (Pediococcus) halophilus ATCC33315. *Mol Gen Genet.* 1997;**255**(5):504–13. [PubMed: 9294035].
15. Bruand C, Le Chatelier E, Ehrlich SD, Janniere L. A fourth class of theta-replicating plasmids: the pAM beta 1 family from gram-positive bacteria. *Proc Natl Acad Sci U S A.* 1993;**90**(24):11668–72. [PubMed: 8265606].
16. Meijer WJ, de Boer AJ, van Tongeren S, Venema G, Bron S. Characterization of the replication region of the Bacillus subtilis plasmid pLS20: a novel type of replicon. *Nucleic Acids Res.* 1995;**23**(16):3214–23. [PubMed: 7667098].
17. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol.* 2007;**24**(8):1596–9. doi: 10.1093/molbev/msm092. [PubMed: 17488738].
18. Zhang H, Hao Y, Zhang D, Luo Y. Characterization of the cryptic plasmid pTXW from Lactobacillus paracasei TXW. *Plasmid.* 2011;**65**(1):1–7. doi: 10.1016/j.plasmid.2010.08.002. [PubMed: 20709099].
19. Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, et al. CDD: NCBI's conserved domain database. *Nucleic Acids Research.* 2014;**43**(D1):D222–6. doi: 10.1093/nar/gku1221.
20. Fukao M, Oshima K, Morita H, Toh H, Suda W, Kim SW, et al. Genomic analysis by deep sequencing of the probiotic Lactobacillus brevis KB290 harboring nine plasmids reveals genomic stability. *PLoS One.* 2013;**8**(3):e60521. doi: 10.1371/journal.pone.0060521. [PubMed: 23544154].
21. Benachour A, Frere J, Boutibonnes P, Auffray Y. Characterization and replication mode determination of the minimal replicon of Tetragenococcus halophila ATCC33315 plasmid pUCL287. *Biochimie.* 1995;**77**(11):868–74. [PubMed: 8824766].
22. Danielsen M. Characterization of the tetracycline resistance plasmid pMD5057 from Lactobacillus plantarum 5057 reveals a composite structure. *Plasmid.* 2002;**48**(2):98–103. [PubMed: 12383727].
23. Motlagh A, Bukhtiyarova M, Ray B. Complete nucleotide sequence of pSMB 74, a plasmid encoding the production of pediocin AcH in Pediococcus acidilactici. *Lett Appl Microbiol.* 1994;**18**(6):305–12. [PubMed: 7764941].
24. Teresa Alegre M, Rodriguez MC, Mesas JM. Characterization of pRS5: a theta-type plasmid found in a strain of Pediococcus pentosaceus isolated from wine that can be used to generate cloning vectors for lactic acid bacteria. *Plasmid.* 2009;**61**(2):130–4. doi: 10.1016/j.plasmid.2008.10.002. [PubMed: 19027788].
25. Nakamura M, Ogata K, Nagamine T, Tajima K, Matsui H, Benno Y. The replicon of the cryptic Plasmid pSBO1 isolated from Streptococcus bovis JB1. *Curr Microbiol.* 2001;**43**(1):11–6. doi: 10.1007/s002840010252. [PubMed: 11375657].
26. Crutz-Le Coq AM, Zagorec M. Vectors for Lactobacilli and other Gram-positive bacteria based on the minimal replicon of pRV500 from Lactobacillus sakei. *Plasmid.* 2008;**60**(3):212–20. doi: 10.1016/j.plasmid.2008.08.002. [PubMed: 18789962].
27. Van Houdt R, Leplae R, Lima-Mendez G, Mergeay M, Toussaint A. Towards a more accurate annotation of tyrosine-based site-specific

recombinases in bacterial genomes. *Mob DNA.* 2012;**3**(1):6. doi: 10.1186/1759-8753-3-6. [PubMed: 22502997].
28. Sorvig E, Skaugen M, Naterstad K, Eijsink VG, Axelsson L. Plasmid p256 from Lactobacillus plantarum represents a new type of replicon in lactic acid bacteria, and contains a toxin-antitoxin-like plasmid maintenance system. *Microbiology.* 2005;**151**(Pt 2):421–31. doi: 10.1099/mic.0.27389-0. [PubMed: 15699191].
29. Heiss S, Grabherr R, Heinl S. Characterization of the Lactobacillus plantarum plasmid pCD033 and generation of the plasmid free strain L. plantarum 3NSH. *Plasmid.* 2015;**81**:9–20. doi: 10.1016/j.plasmid.2015.05.004. [PubMed: 26038184].
30. Jahn M, Vorpahl C, Hubschmann T, Harms H, Muller S. Copy number variability of expression plasmids determined by cell sorting and Droplet Digital PCR. *Microb Cell Fact.* 2016;**15**(1):211. doi: 10.1186/s12934-016-0610-8. [PubMed: 27993152].
31. Tomizawa J, Itoh T. Plasmid ColE1 incompatibility determined by interaction of RNA I with primer transcript. *Proc Natl Acad Sci U S A.* 1981;**78**(10):6096–100. [PubMed: 6171811].
32. Brantl S. Plasmid Replication Control by Antisense RNAs. *Microbiol Spectr.* 2014;**2**(4):PLAS–1-2013. doi: 10.1128/microbiolspec.PLAS-0001-2013. [PubMed: 26104196].
33. Dong L, Meng Y, Sui Z, Wang J, Wu L, Fu B. Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material. *Sci Rep.* 2015;**5**:13174. doi: 10.1038/srep13174. [PubMed: 26302947].
34. Jahn M, Vorpahl C, Turkowsky D, Lindmeyer M, Buhler B, Harms H, et al. Accurate determination of plasmid copy number of flow-sorted cells using droplet digital PCR. *Anal Chem.* 2014;**86**(12):5969–76. doi: 10.1021/ac501118v. [PubMed: 24842041].

Table 2. Putative Genes and Their Products, Deduced from the Plasmid Nucleotide Sequences

ORF	Position in Nucleotide Sequence, bp		G + C	Protein		Closest Relative (length, Level of Amino Acid Identity, Micro-Organism)	Accession no.
	5'	3'	%	Length, aa	Molecular mass, Da		
pBM1							
orf1	1820	1332	37.01	162	18640.6	Hypothetical protein (162aa, 97%, *Lactobacillus plantarum*)	WP_015063543.1
orf2	1933	2295	36.09	120	13825.7	Transcriptional regulator (112aa, 96%, *Lactobacillus plantarum*)	WP_011031954.1
orf3	2772	2416	36.97	118	13847.5	Addiction module toxin RelE (plasmid) (118aa, 99, %*Lactobacillus plantarum* HFC8)	ALG27536.1
						Addiction module toxin, RelE/StbE family (plasmid) (118aa, 98%, *Lactobacillus buchneri* NRRL B-30929)	AEB74676.1
orf4	3050	2772	35.48	92	10708.7	Prevent-host-death family protein (93aa, 100%, *Lactobacillus brevis*)	WP_042253922.1
						Antitoxin (plasmid) (92aa, 98%, *Lactobacillus plantarum* HFC8)	ALG27537.1
orf5	3127	3714	40.65	195	22480.7	Integrase (195aa, 99%, *Lactobacillus pentosus* IG1)	CCC15493.1
						Integrase (195aa, 100%, *Lactobacillus plantarum* HFC8)	ALG27531.1
orf6	3727	4086	35.28	119	14235.3	Hypothetical protein (119aa, 100%, *Lactobacillus plantarum*)	WP_016511831.1
						Hypothetical protein (119aa, 98%, *Lactobacillus brevis*)	WP_015474693.1

orf7	4483	4695	31.46	70	8288.3	Hypothetical protein (70aa, 99%, *Lactobacillus versmoldensis*)	WP_010625611.1
						Hypothetical protein (70aa, 97%, *Lactobacillus plantarum*)	WP_027821986.1
orf8	5786	5283	46.63	167	18712.4	Hypothetical protein (167aa, 99%, *Lactobacillus plantarum* P-8)	AGL65684.2
						Transposase (167aa, 98%, *Lactobacillus paraplantarum*)	CDF77674.1
pBM2							
orf1	588	1	40.48	195	22538.9	Putative integrase/recombinase (195aa, 99%, *Lactobacillus casei*)	WP_003586668.1
						Integrase (195aa, 96%, *Lactobacillus sakei* KCA311)	AJQ16980.1
orf2	667	930	37.88	87	9981.6	AbrB family transcriptional regulator (plasmid) (87aa, 100%, *Lactobacillus plantarum* HFC8)	ALG27235.1
orf3	930	1277	39.66	115	13031.8	PemK family protein (115aa, 96%, *Lactobacillus plantarum* HFC8)	ALG27234.1
orf4	2063	1695	41.46	122	12265.3	Prophage Lp1 protein 6 (122aa, 100%, *Lactobacillus pentosus* MP-10)	CCB84218.1
						Membrane protein (122aa, 99% , *Lactobacillus plantarum*)	WP_011101080.1
orf5	2397	2143	31.76	84	10054.2	Hypothetical protein (84aa, 99%, *Lactobacillus plantarum*)	WP_016527215.1
orf6	2871	2407	37.63	154	17395.3	Hypothetical protein (263aa, 98%, *Lactobacillus plantarum*)	WP_024002855.1

orf7	3350	3853	46.63	167	18712.4	Hypothetical protein (167aa, 99%, *Lactobacillus plantarum* subsp. *plantarum* P-8)	AGL65684.2
						Transposase (167aa, 98%, *Lactobacillus paraplantarum*)	CDF77674.1
repB	4829	4314	35.08	171	19956.9	Replication protein RepB (171aa, 84%, *Lactobacillus brevis*)	WP_041816333.1
						Replication protein RepB (171aa, 84%, *Lactobacillus brevis* KB290)	BAN08207.1
						Replication protein (192aa, 66%, *Pediococcus acidilactici* H plasmid pSMB74)	AAP55633.1
repA	5814	4822	34.74	330	38652.7	Initiator Replication family protein (311aa, 99%, *Pediococcus pentosaceus*)	WP_002834578.1
						Probable replication protein rep (plasmid) (376aa, 99%, *Lactobacillus brevis* KB290)	BAN08206.1
						Rep3 (plasmid pSMB74) (319aa, 82%, *Pediococcus acidilactici*)	CAA53278.1
						Replication protein A (plasmid pMD5057) (311aa, 81%, *Lactobacillus plantarum* 5057)	AAP55632.1
orf10	6688	6476	32.86	70	8231.2	Hypothetical protein (70aa, 97%, *Lactobacillus nodensisi*)	WP_025025371.1
						Hypothetical protein (70aa, 97%, *Lactobacillus versmoldensis*)	WP_010625611.1
pBM3							
orf1	766	554	32.8	70	8369.7	Hypothetical protein (70aa, 99%, *Lactobacillus brevis*)	WP_042253900.1

						Hypothetical protein (70aa, 96%, *Lactobacillus nodensis*)	WP_025025371.1
orf2	1522	1163	36.1	119	14162.1	Hypothetical protein (119aa, 99%, *Lactobacillus plantarum*)	WP_027821987.1
						Hypothetical protein (plasmid) (121aa, 99%, *Lactobacillus brevis* BSO 464)	AJA81476.1
orf3	2122	1535	41	195	22475.8	Integrase (195aa, 91%, *Lactobacillus pentosus* IG1)	CCC15451.1
						Integrase (195aa, 99%, *Lactobacillus brevis*)	WP_011669005.1
orf4	2213	2494	36.5	93	10822.1	Antitoxin (93aa, 100% , *Lactobacillus brevis*)	WP_011669006.1
						Antitoxin (pMK06) (92aa, 96%, *Lactobacillus plantarum* HFC8)	ALG27537.1
orf5	2491	2847	37.5	118	13861.9	Plasmid stabilisation system protein (118aa, 100% , *Lactobacillus brevis*)	WP_011669007.1
						Addiction module toxin RelE (pMK06) (118aa, 97%, *Lactobacillus plantarum* HFC8)	ALG27529.1
orf6	2923	3300	36.7	125	14714.5	Transposase, partial (112aa, 93% , *Lactobacillus plantarum*)	WP_046041026.1
orf7	5296	3602	31.5	564	65233.9	Cell surface protein (564aa, 99%, *Lactobacillus plantarum*)	WP_016527444.1
orf8	5812	6300	37.8	162	18792	Hypothetical protein (162aa, 95% , *Lactobacillus plantarum*)	WP_016511466.1
						Hypothetical protein (162aa, 94%, *Lactobacillus rhamnosus*)	WP_024306043.1

orf9	7005	7385	37.3	126	14408.6	Hypothetical protein (125aa, 97%, *Lactobacillus plantarum*)	WP_-003646093.1
						Hypothetical protein (125aa, 86%, *Lactobacillus plantarum*WJL)	ERO39647.1
orfi10	7848	7345	46.8	167	18744.6	Transposase (167aa, 99%, *Lactobacillus plantarum* subsp. *plantarum* P-8)	AGL65684.2
						Transposase (167aa, 99%, *Lactobacillus plantarum*)	WP_024272162.1
pBM4							
orf1	662	129	39.89	177	20171.7	Transposase (177aa, 99%, *Lactobacillus plantarum*)	WP_021731278.1
						Transposase (177aa, 91%, *Lactobacillus paracasei*)	WP_016385682.1
orf2	1936	1106	28.28	276	32479.2	Endonuclease (278 aa, 69%, *Lactobacillus brevis*)	WP_042749360.1
orf3	2456	1998	28.1	152	17600.8	Hypothetical protein (152aa, 68%, *Lactobacillus plantarum*)	WP_046947582.1
orf4	4043	3540	38.89	167	19284.3	Hypothetical protein (167aa, 98%, *Lactobacillus plantarum*)	WP_011031951.1
						Hypothetical protein (167aa, 91%, *Lactobacillus plantarum*)	WP_045353452.1
orf5	4867	4400	43.59	155	18007.2	Ferritin (155aa,100%, *Lactobacillus brevis*)	WP_042253884.1
						Stress induced DNA binding protein (155aa, 99%, *Lactobacillus sakei* KCA311)	AJQ16931.1
orf6	6633	5422	28.22	403	46440.9	Hypothetical protein (401aa, 75%, *Lactobacillus florum*)	WP_009166650.1
orf7	7097	6795	33.33	100	11858.1	Translation repressor RelE (100aa, 97%, *Lactobacillus plantarum*)	WP_021357787.1

						Translation repressor RelE (100aa, 99%, *Lactobacillus versmoldensis*)	WP_010625626.1
orf8	7365	7087	36.92	92	10106	RelB (92aa, 98%, *Lactobacillus plantarum*)	WP_027822890.1
orf9	7469	8056	38.44	195	22447	Integrase (195aa, 99%, *Lactobacillus brevis*)	WP_042253402.1
						Integrase (195aa, 96%, *Lactobacillus pentosus* MP-10)	CCB81910.1
orf10	8480	8692	31.92	70	8273.3	Hypothetical protein (70aa, 96%, *Lactobacillus nodensis*)	WP_025025371.1
						Hypothetical protein (70aa, 96%, *Lactobacillus brevis*)	WP_042253900.1