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Isolation and Characterization of a Cryptic Plasmid, pMBLR00, from *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3733

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A cryptic plasmid, pMBLR00, from *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3733 was isolated, characterized, and used for the construction of a cloning vector to engineer *Leuconostoc* species. pMBLR00 is a rolling circle replication plasmid, containing 3,370 base pairs. Sequence analysis revealed that pMBLR00 has 3 open reading frames: Cop (copy number control protein), Rep (replication protein), and Mob (mobilization protein). pMBLR00 replicates by rolling circle replication, which was confirmed by the presence of a conserved double-stranded origin and single-stranded DNA intermediates. An *Escherichia coli–Leuconostoc* shuttle vector, pMBLR02, was constructed and was able to replicate in *Leuconostoc citreum* 95. pMBLR02 could be a useful genetic tool for metabolic engineering and the genetic study of *Leuconostoc* species.

Key words: Cryptic plasmid, *Leuconostoc mesenteroides*, rolling circle replication mechanism, D-lactic acid

Lactic acid bacteria (LAB) are a non-taxonomic group of Gram-positive, low G+C content bacteria. LAB include *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus, Streptococcus*, and *Bifidobacterium* species. LAB are used for food and beverage fermentation, for grass silage, and as bacteriocins [11]. The recent discovery of biodegradable poly-L,D-lactic acid (L,D-PLA) has boosted the importance of LAB and lactic acid production [29, 33]. Owing to the growing market for the biodegradable and renewable polymer L,D-PLA, the world demand for lactic acid, especially D-lactic acid [18], is rapidly increasing.

Many cryptic plasmids originating from LAB species have been isolated and characterized [26, 32]. Based on biochemical evidence and sequence similarity studies of the origins of replication and the replication proteins, it has been speculated that a large number of plasmids exist in LAB [12, 14]. Moreover, vectors based on these plasmids have been developed and used to clone and express several heterologous genes [1, 17, 21, 22, 24].

Leuconostoc mesenteroides species is the most important microorganism for *kimchi* fermentation [34]. They are regarded as promising producers of D-lactic acid [10]. However, even with growing attention to D-lactic acid producers, there have been only a few reports characterizing plasmids from *Leuconostoc* species [9, 13]. Because of the industrial application of *Leuconostoc mesenteroides* for D-lactic acid production, it is important to develop a reproducible genetic system that can be used to genetically and metabolically engineer this bacterium.

In this study, we report on a novel cryptic plasmid named pMBLR00 with a rolling circle replication (RCR) mode. Based on the sequence information on pMBLR00, a *Leuconostoc–Escherichia coli* shuttle vector was constructed and characterized. This vector will be useful in the metabolic engineering of *Leuconostoc* species for the mass production of D-lactic acid.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Leuconostoc* strains were routinely grown on MRS medium (Difco Laboratories, USA). Recombinant *L. citreum* 95 harboring a shuttle vector was cultivated in a 125 ml serum bottle containing MRS medium supplemented with 2% (w/v) $CaCO_3$ (BioBasic, Canada) for 24 h at 30°C without shaking. *E. coli* JM109 was used as a host for recombinant plasmids and preparation of sequencing templates [20]. *E. coli* was grown in Luria–Bertani (LB) medium at 37°C with vigorous shaking. When appropriate, antibiotics were added as follows: ampicillin at 100 µg/ml for *E. coli* and erythromycin at 20 µg/ml for *Leuconostoc* strains.

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Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Source or reference[b]
Escherichia coli		
JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14- [F' traD36 proAB+ lacIq lacZΔM15] hsdR17(rK-mK+)	NEB
Lactic acid bacteria		
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC 3733	Wild-type, host of pMBLR00	KCTC
Leuconostoc citreum 95	Wild-type *kimchi* isolate	[15]
Plasmids		
pUC19	Cloning vector, Ap^r	NEB
pUC19-3733lib1	2.2 kbp *Hin*dIII fragment of pMBLR00 cloned in pUC19	This study
pUC19-3733lib2	1.2 kbp *Hin*dIII fragment of pMBLR00 cloned in pUC19	This study
pTOPO-Em	TA cloning vector, containing *erm* gene, Ap^r	Invitrogen
pMBLR00	Cryptic plasmid from *L. mesenteroides* (KCTC 3733)	This study
pMBLR01	*Leuconostoc*–*E. coli* shuttle vector, Ap^r	This study
pMBLR02	*Leuconostoc*–*E. coli* shuttle vector, Ap^r, Em^r	This study

[a]Ap^r, ampicillin resistance; Em^r, erythromycin resistance.
[b]KCTC, Korean Collection for Type Cultures.

DNA Manipulation Techniques

General procedures for DNA manipulation were conducted as described by Sambrook and Russell [28]. Plasmid DNAs were isolated from *E. coli* by alkaline lysis and purified by using a DNA-spin plasmid purification kit according to the manufacturer's instructions (iNtRON, Korea). Plasmid DNAs from *L. mesenteroides* KCTC 3733 were isolated with the extra step of adding lysozyme (Sigma, USA) at 15 mg/ml and incubating at 37°C for 1 h. The smallest plasmid, pMBLR00, was isolated from an agarose gel following agarose gel electrophoresis of *L. mesenteroides* plasmid DNAs. Restriction endonuclease, T4 DNA ligase, and Vent DNA polymerase were purchased commercially and used according to the recommendations of the supplier (NEB, USA). Electroporation was used for plasmid transfer into *E. coli* and *Leuconostoc citreum* 95 as previously described [4]. Transformants were selected using LB or MRS agar containing the appropriate antibiotics.

Plasmid DNA Sequencing and Bioinformatics Analysis

To determine the nucleotide sequence, pMBLR00 was digested with restriction enzymes and then cloned into the vector pUC19. The recombinant plasmid DNA was sequenced with primers 5'-CCG ACTGGAAAGCGG-3' (forward), 5'-ACAAGCCCGTCAGGG-3' (reverse), and several walking primers (Macrogen, Korea). The DNA and amino acid sequences were handled and analyzed using the DNASTAR (DNASTAR, USA) and Artemis12 programs [7]. Open reading frames (ORFs) were predicted with the GeneMark [5] and FGENESB (SoftBerry, USA) programs. Gene annotation and similarity analysis were performed using BLAST programs of the National Center for Biotechnology Information (NCBI) [2]. The conserved functional protein domain analysis of the predicted ORFs was conducted using the InterProScan program of the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and the NCBI Conserved Domain Database (CDD) [23]. Multiple sequence alignment analysis was performed using the BLAST2SEQ [30] and ClustalW [31] programs. DNA repeats were detected using the Tandem Repeats Finder [3] and the EMBOSS package [27] programs.

Construction of a Shuttle Vector

Plasmid pMBLR00 was linearized with *Sac*I, and the molecule was cloned into the *E. coli* plasmid vector pUC19, resulting in pMBLR01. In addition, the erythromycin resistance gene, *erm*, from pTOPO-Em was amplified by PCR with primers containing *Eco*RI sites (Table 2). The *erm* gene was inserted into the *Eco*RI site of pMBLR01, resulting in pMBLR02.

Table 2. Primers used in this study.

Gene	Sequence[a]	Enzyme site
erm	F:5' ccggaattcaggaggattacaaaataaggaggaaaaaatatgg	*Eco*RI
	R:3' ccggaattctggtttaagccgactgc	*Eco*RI
mob_R (pMBLR00 probe)	F:5' gtgcgtactatgagtt	
	R:3' aagatgtggaccgcct	
mob_T (pMBLT00 probe)	F:5' gtgttatactggtctc	
	R:3' aggacttcaacgacta	

[a]Restriction enzyme sites are underlined.

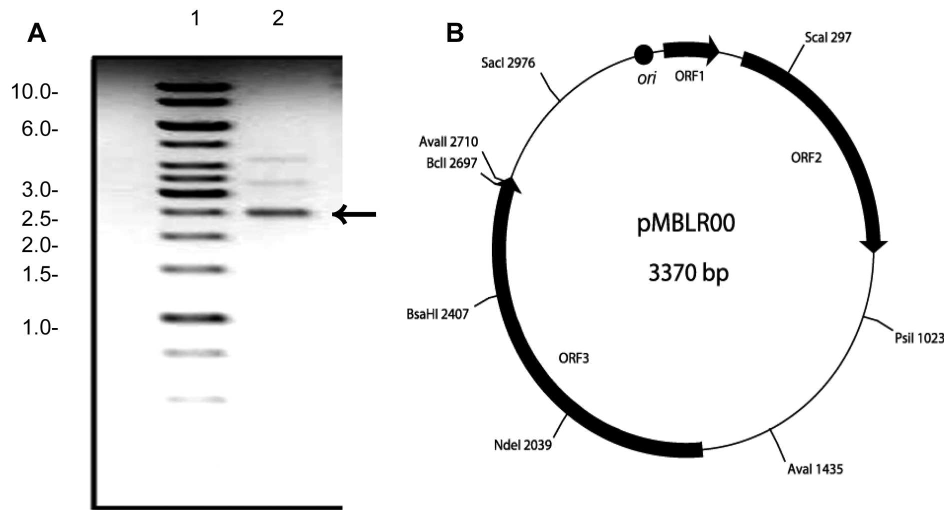

Fig. 1. Isolation and map of plasmid pMBLR00.

(**A**) Agarose gel electrophoresis of the cryptic plasmid pMBLR00 isolated from *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3733. (**B**) Three open reading frames and unique restriction enzyme maps of plasmid pMBLR00. Lane 1, 1 kb DNA size marker; lane 2, pMBLR00 (indicated by an arrow). The black-filled circle indicates the origin of replication (*ori*).

Southern Hybridization

Bacteria were harvested in exponential phase after culture in MRS medium, and total DNA was extracted as previously described [8]. Single-stranded DNA (ssDNA) was detected by using a mung bean nuclease (NEB, USA) treatment. Mung bean nuclease treatment was carried out at 37°C for 30 min. The treated DNA was electrophoresed in 0.7% (w/v) agarose gels and transferred to nylon membranes without denaturation treatment. The *mob* genes (mob_R for pMBLR00 and mob_T for pMBLT00 [10]), used as probes, were amplified by PCR with the primers listed in Table 2. Labeling and detection were performed using a DIG Labeling and Detection kit (Roche, USA) according to the manufacturer's instructions.

Nucleotide Sequence Accession Number

The pMBLR00 nucleotide sequence reported in this paper was submitted to GenBank with the accession number JN106353.

RESULTS AND DISCUSSION

Isolation of Cryptic Plasmid pMBLR00

The smallest plasmid found in *L. mesenteroides* KCTC 3733 (Fig. 1A), designated pMBLR00, was isolated and digested with several commonly used restriction enzymes (REs) to determine the appropriate RE sites (data not shown). Digestion of pMBLR00 with *Hin*dIII produced 2 DNA fragments, 2.2 and 1.2 kilobase pairs (kbp) long, which were separately subcloned into the *Hin*dIII site of a pUC19 vector and then sequenced.

Sequence Analysis of pMBLR00

The complete plasmid sequence of pMBLR00 consists of 3,370 base pairs (bps) with a G+C content of 36.5%, which is within the known range (31–39%) of *Leuconostoc* species chromosomes and plasmids [6, 9, 25]. In this plasmid, 3 ORFs encoding a plasmid copy number control gene (*cop*), a plasmid replication gene (*rep*), and a mobilization gene (*mob*) were predicted by bioinformatics programs (Table 3). The putative protein encoded by ORF1 exhibited 100% identity with a copy number control protein encoded by *L. sakei* plasmid pYSI8 (Table 3). Plasmid pYS18 was identified as a member of the pMV158 family of rolling circle plasmids [35]. Thus, it is plausible that pMBLR00 belongs to the same family. A copy number control Cop protein has been found in some

Table 3. ORF analysis of the pMBLR00 plasmid.

ORF	Function	Position (amino acids)	% Identities	Best BLASTP match	GenBank No.
ORF1	Copy number control protein	3308–93 (51)	100	CopG: Copy number control protein from *L. sakei* plasmid pYSI8	ABW71676
ORF2	Replication protein	166–849 (227)	99	Rep2: Plasmid replication protein from *L. sakei* plasmid pYSI8	ABW71677
ORF3	Mobilization protein	1639–2724 (361)	89	Mob: Mobilization protein from *L. plantarum* plasmid pPLA4	ABG23031

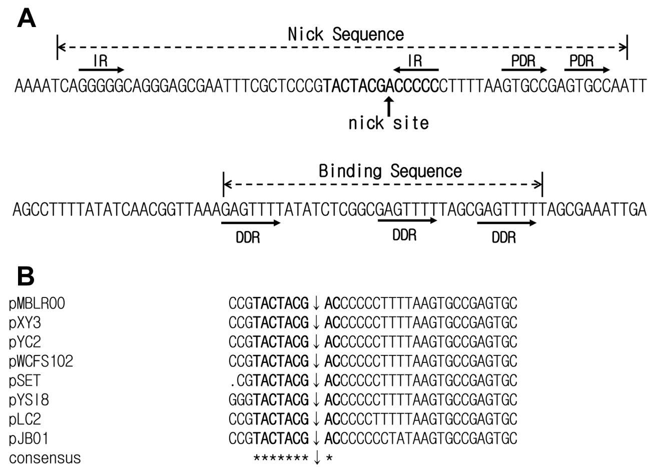

Fig. 2. pMBLR00 nick and binding sequences and comparison with other plasmid nick sequences.

(**A**) Double-strand origin (*dso*) sequence in pMBLR00. The nick sequence and binding sequence are shown with inverted repeats (IR), proximal direct repeats (PDR), and distal direct repeats (DDR) indicated by arrows. The nick site is indicated by an arrow between G and A. (**B**) Comparison of a putative nick site sequence with sequences in other closely related plasmids. Arrows indicate nick sites and asterisks indicate conserved nucleotides.

plasmids from specific genera such as *Lactobacillus* and *Streptococcus*. This putative protein has a major conserved protein domain, a Cop-like DNA binding domain (PF01402), which is involved in binding the Rep protein promoter for regulation of plasmid copy number [16]. The double-strand origin (*dso*) of replication of rolling circle plasmids is functionally divided into 2 regions: a nick region and a binding region, where the Rep protein binds specifically [19]. The nick and binding sequences are highly conserved in pMBLR00 (Fig. 2A), suggesting that this plasmid replicates by the RCR mechanism (see next section for details). In particular, the nick site sequence is highly conserved and almost identical to nick site sequences from other closely related plasmids [35] (Fig. 2B).

The predicted protein from ORF2 is highly similar to other *Lactobacillus* plasmid Rep proteins containing a Rep_2 plasmid replication protein domain (PF01719). Interestingly, the origin of replication (*ori*) and a putative CopG protein (ORF1) are present upstream of this Rep protein, which is very specific for *Lactobacillus* and some *Streptococcus* plasmids. This organization characteristic is similar to that of the pMV158 family [12]. Furthermore, phylogenetic analysis of the Rep protein in this plasmid showed that this Rep protein is very similar to other Rep proteins in *Lactobacillus* plasmids that replicate by the RCR mechanism (Fig. 3).

ORF3 was located at nucleotides 1,639 to 2,724, and the gene product is homologous to mobilization proteins encoded by plasmids isolated from *Leuconostoc* and *Lactobacillus* species. The best identity score (89%) was

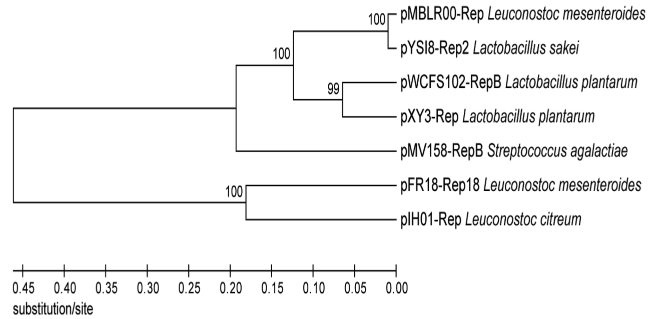

Fig. 3. Phylogenetic analysis of the Rep protein in the pMBLR00 plasmid and rolling circle replication Rep proteins in other closely related plasmids using ClustalW multiple alignments.

A phylogenetic tree was constructed using the neighbor-joining method and *P* distances. The numbers associated with the branches represent the bootstrap values.

observed for the mobilization protein (Mob) of *L. plantarum* plasmid pPLA4 (Table 3). This putative mobilization protein has a major conserved domain: a plasmid mobilization/recombination enzyme domain (PF01076). According to the classification criteria of Mob proteins [15], the pMBLR00 Mob protein belongs to the MOBv family, based on conserved motif I (HNQR) and motif II (AQVHLDETTPHMHLG). Like other typical Mob proteins, a highly conserved *oriT* sequence consisting of a conserved inverted repeat is present in pMBLR00 (Fig. 4). In addition to the high similarity of pMBLR00 Mob protein to Mob proteins of other *Lactobacillus* plasmids, the pMBLR00 Mob *oriT* sequence is also almost identical to the *oriT* sequences of a *Lactobacillus* plasmid (pYS18), a streptococcal plasmid (pMV158), and 2 *Staphylococcus* plasmids (pSCFS1 and pSES22), suggesting that these plasmids may share a plasmid transfer mechanism.

In addition to the high similarity of the 3 ORFs and *oriT* structure to those of *Lactobacillus* plasmids, BLASTN analysis of the pMBLR00 DNA sequence revealed that the sequence is highly homologous to other *Lactobacillus*

Fig. 4. Comparative analysis of the putative transfer origin (*oriT*) upstream of the Mob protein and putative *oriT* sequences in other closely related plasmids.

Asterisks indicate conserved nucleotides in the putative *oriT* structure. Arrows indicate inverted repeats.

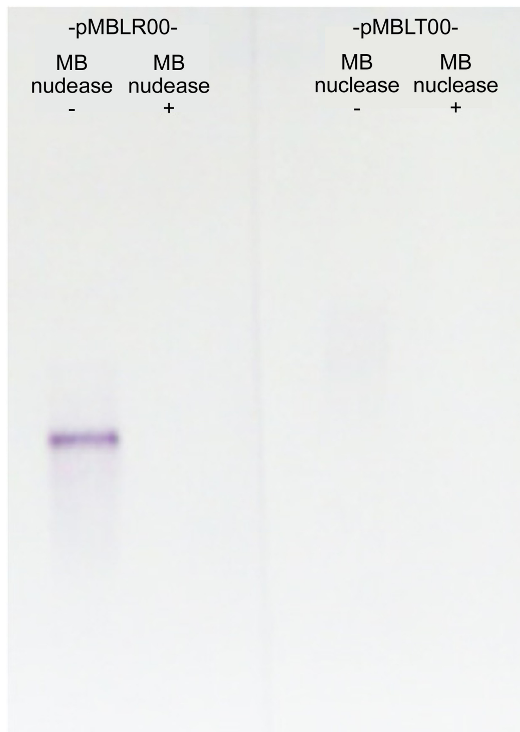

Fig. 5. Mung bean nuclease analysis of the pMBLR00 plasmid and a theta-replicating pMBLT00 plasmid (for comparison) under non-denaturation conditions to investigate the existence of single-stranded DNA intermediates.

MB nuclease –, not treated with mung bean nuclease; MB nuclease +, treated with mung bean nuclease.

plasmids at the DNA level, suggesting that this plasmid probably shares a common ancestor with *Lactobacillus* plasmids

Detection of Single-Stranded Plasmid DNA by Southern Hybridization

The ssDNA intermediate is a hallmark of the RCR mechanism [12]. Therefore, detection of ssDNA by Southern hybridization was used to confirm the plasmid replication mechanism. Analysis of the replication mode using Mung bean nuclease digestion revealed the accumulation of single-stranded DNA of pMBLR00, substantiating that the pMBLR00 plasmid uses the RCR mechanism for replication (Fig. 5). A control theta-replicating plasmid, pMBLT00 [8], did not show a positive band (Fig. 5). This result is consistent with the presence of a nick site in the *ori* region and the high homology of the Rep protein in pMBLR00 to Rep proteins in RCR plasmids.

Construction of *Leuconostoc–E. coli* Shuttle Vector pMBLR02

To construct a *Leuconostoc–E. coli* shuttle vector, plasmid pMBLR00 was linearized with *Sac*I and cloned into the *E. coli* plasmid vector pUC19, resulting in pMBLR01. As a selection marker, an erythromycin resistance gene (*erm*) was cloned into the *Eco*RI site of pMBLR01, resulting in pMBLR02. pMBLR02 was electroporated into *Leuconostoc citreum* 95 to investigate its replication. pMBLR02 was able to replicate in the recombinant strain, suggesting that the *cop* and *rep* gene products were functional for plasmid replication in the heterologous strain.

In summary, nucleotide sequence analysis of pMBLR00 from *Leuconostoc mesenteroides* KCTC 3733 revealed that pMBLR00 has 3 open reading frames: a copy number control protein, a replication protein, and a mobilization protein. pMBLR00 replicates by rolling circle replication, which was confirmed by the presence of a conserved double-stranded origin and single-stranded DNA intermediates. The development of RCR-type pMBLR02 will provide further insight into plasmid transfer between different bacteria and facilitate metabolic engineering in major lactic acid-producing bacteria.

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