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-D,L-lactic acid (D,L-PLA) has boosted the importance of LAB and lactic acid production [29, 33]. Owing to the growing market for the biodegradable and renewable polymer D,L-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) CaCO₃ (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.
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* 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 SacI, 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 EcoRI sites (Table 2). The *erm* gene was inserted into the EcoRI site of pMBLR01, resulting in pMBLR02.

### Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14- [F’ traD36 proAB+ lacIq lacZAΔM15] hsdR17(R-K-mK+)</td>
<td>NEB</td>
</tr>
<tr>
<td><strong>Lactic acid bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Leuconostoc mesenteroides</em> subsp. <em>mesenteroides</em> KCTC 3733</td>
<td>Wild-type, host of pMBLR00</td>
<td>KCTC</td>
</tr>
<tr>
<td><em>Leuconostoc citreum</em> 95</td>
<td>Wild-type <em>kimchi</em> isolate</td>
<td>[15]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector, Ap'</td>
<td>NEB</td>
</tr>
<tr>
<td>pUC19-3733lib1</td>
<td>2.2 kbp HindIII fragment of pMBLR00 cloned in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19-3733lib2</td>
<td>1.2 kbp HindIII fragment of pMBLR00 cloned in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pTOPO-Em</td>
<td>TA cloning vector, containing <em>erm</em> gene, Ap’</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMBLR00</td>
<td>Cryptic plasmid from <em>L. mesenteroides</em> (KCTC 3733)</td>
<td>This study</td>
</tr>
<tr>
<td>pMBLR01</td>
<td><em>Leuconostoc</em>-<em>E. coli</em> shuttle vector, Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pMBLR02</td>
<td><em>Leuconostoc</em>-<em>E. coli</em> shuttle vector, Ap’, Em’</td>
<td>This study</td>
</tr>
</tbody>
</table>

*a*, Ap', ampicillin resistance; *Em’, erythromycin resistance.

**KCTC**, Korean Collection for Type Cultures.

### Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence*</th>
<th>Enzyme site</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>erm</em></td>
<td>F:5' ccggaattcaggaggattaaataaggaaagaaaaatatgg</td>
<td>EcoRI</td>
</tr>
<tr>
<td></td>
<td>R:3' ccggaattcggtagggactgc</td>
<td>EcoRI</td>
</tr>
<tr>
<td><em>mob</em>&lt;sub&gt;b&lt;/sub&gt;</td>
<td>F:5' gtgtgactatatggtt</td>
<td></td>
</tr>
<tr>
<td>(pMBLR00 probe)</td>
<td>R:3' aagatgtgaggcgctt</td>
<td></td>
</tr>
<tr>
<td><em>mob</em>&lt;sub&gt;1&lt;/sub&gt;</td>
<td>F:5' ggtttatactggtcte</td>
<td></td>
</tr>
<tr>
<td>(pMBLT00 probe)</td>
<td>R:3' aggcaattcaagacta</td>
<td></td>
</tr>
</tbody>
</table>

*Restriction enzyme sites are underlined.*
CHARACTERIZATION OF A CRYPTIC PLASMID pMBLR00

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 (mobR for pMBLR00 and mobT 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 HindIII produced 2 DNA fragments, 2.2 and 1.2 kilobase pairs (kbp) long, which were separately subcloned into the HindIII 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 pYS18 (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.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Function</th>
<th>Position (amino acids)</th>
<th>% Identities</th>
<th>Best BLASTP match</th>
<th>GenBank No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1</td>
<td>Copy number control protein</td>
<td>3308–93 (51)</td>
<td>100</td>
<td>CopG: Copy number control protein from L. sakei plasmid pYS18</td>
<td>ABW71676</td>
</tr>
<tr>
<td>ORF2</td>
<td>Replication protein</td>
<td>166–849 (227)</td>
<td>99</td>
<td>Rep2: Plasmid replication protein from L. sakei plasmid pYS18</td>
<td>ABW71677</td>
</tr>
<tr>
<td>ORF3</td>
<td>Mobilization protein</td>
<td>1639–2724 (361)</td>
<td>89</td>
<td>Mob: Mobilization protein from L. plantarum plasmid pPLA4</td>
<td>ABG23031</td>
</tr>
</tbody>
</table>

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).
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 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 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*...
CHARACTERIZATION OF A CRYPTIC PLASMID pMBLR00

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 SacI 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 *EcoRI* 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.

Acknowledgments

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REFERENCES