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Structural Analysis of Plasmid pCL2.1 from *Lactococcus lactis* ssp. *lactis* ML_8 and the Construction of a New Shuttle Vector for Lactic Acid Bacteria

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Abstract The nucleotide sequence contains 2 open reading frames encoding a 45-amino-acid protein homologous to a transcriptional repressor protein CopG, and a 203-amino-acid protein homologous to a replication protein RepB. Putative countertranscribed RNA, a double-strand origin, and a single-strand origin were also identified. A shuttle vector, pUCL2.1, for various lactic acid bacteria (LAB) was constructed on the basis of the pCL2.1 replicon, into which an erythromycin-resistance gene as a marker and *Escherichia coli* ColE1 replication origin were inserted. pUCL2.1 was introduced into *E. coli*, *Lc. lactis*, *Lactobacillus* (*Lb.*) *plantarum*, *Lb. paraplantarum*, and *Leuconostoc mesenteroides*. The recombinant LAB maintained traits of transformed plasmid in the absence of selection pressure over 40 generations. Therefore, pUCL2.1 could be used as an *E. coli*/LAB shuttle vector, which is an essential to engineer recombinant LAB strains that are useful for food fermentations.

Keywords: pCL2.1, shuttle vector, lactic acid bacteria

Introduction

Lactic acid bacteria (LAB) are widely used in the food industry for the production and preservation of fermented products such as dairy products (e.g., cheese and yogurt) and vegetable products (e.g., *kimchi* and sauerkraut). The traditional use of these bacteria in the food industry confirms their lack of pathogenicity and their status as generally regarded as safe (GRAS) organisms. LAB have also been used as probiotics to manage intestinal disorders such as lactose intolerance, acute gastroenteritis due to rotaviruses and other enteric pathogens, inflammatory bowel disease, and food allergies (1,2). LAB exhibit antimicrobial, hypercholesterolemic, immune-enhancing, and antitumor activities (2-6). Because of their industrial significance, LAB have become the focus of genetics studies, especially those involving the application of recombinant DNA techniques. Recombinant LAB with novel properties have been developed for applications in the health-care industry and for traditional uses in food fermentation and preservation (7-11). The eventual aim of genetically modified LAB is to produce flavor compounds and enzymes for food production, and serve as cellular factories for the production of antimicrobials and other high-value proteins.

Vectors for the gene expression is one of the required elements for constructing genetically modified LAB with specific purposes. The vector systems for gene expression and the delivery of specific genes can be categorized into replicative and integrative systems (12). Replicative systems provide considerable advantages over integrative systems, such as involving simpler manipulations and efficient gene introduction into LAB. Recombinant LAB have recently been constructed using replicative vector systems based on LAB harboring plasmids (13,14).

Most LAB harbor one or more plasmids, and most such plasmids have been shown to replicate by the rolling-circle mechanism that includes the formation of single-stranded DNA (ssDNA) intermediates. Rolling-circle-replicating (RCR) plasmids are usually small (less than 10 kb), have multiple copies, and are tightly organized. All RCR plasmids contain 3 important elements - a gene encoding the replication-initiation protein (*rep*), a double-strand origin (*dso*), and a single-strand origin (*sso*) (15) - and they usually have a broad host range. These characteristics render RCR plasmids suitable for vector construction and gene cloning. A cryptic plasmid, pCL2.1, from *Lactococcus* (*Lc.*) *lactis* ssp. *lactis* ML_8 was characterized in our laboratory (16). It was a small plasmid that originated from a food micro-organism, and satisfied some of the requirements for a food-grade vector for use in the food industry. We considered the construction of a new food-grade vector based on the pCL2.1 frame. Two open reading frames (ORF1 and ORF2) were identified in pCL2.1. ORF1 showed no significant homology with proteins in GenBank, whereas the nucleotide sequence of ORF2 showed 81% identity with partial nucleotide sequences of *repB* in pCI411 (NCBI accession No.: NC_004992) from *Leuconostoc* (*Leu.*) *lactis* 533 (17), and the translated amino acid sequence showed 92% identity. Despite the tremendous increase in the amount of DNA and protein data over the past decade, a protein that exhibits significant homology with the translated ORF1 has not been found, which raised a possibility that the initial sequence data might have some errors. Therefore, in the present study we reanalyzed the nucleotide sequence of pCL2.1 and used this plasmid to

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construct a shuttle cloning vector for use in the genetic engineering of LAB.

Materials and Methods

Bacterial strains, plasmids, and culture conditions *Escherichia coli* DH5α was used for plasmid construction. *Lc. lactis* MG1363 (18), *Lactobacillus* (*Lb.*) *plantarum* KCTC 3109 (from the Korean Collection for Type Cultures), *Lb. paraplantarum* C7 (19), and *Leu. mesenteroides* C7 (20) were used as hosts. *E. coli* EC101 was grown in LB medium at 37°C, *Lc. lactis* MG1363 was grown in M17 medium (Difco, Detroit, MI, USA) supplemented with 0.5%(w/v) glucose at 30°C, and the other LAB were cultivated in MRS medium (Difco). Ampicillin (100 mg/mL) and erythromycin (200 mg/mL) were employed as antibiotics for *E. coli*, and erythromycin (5 mg/mL) was used for LAB. pUC18 (NEB, Beverly, MA, USA) was used as a vector for ligating pCL2.1, and pIL253 was used as a donor plasmid of the erythromycin gene (21).

Sequence analysis of pCL2.1 Eight oligonucleotide primers based on the previously reported nucleotide sequence of pCL2.1 (NCBI accession No.: U26594.1) were constructed and used for DNA sequencing in order to confirm the sequence of the plasmid. DNA and amino acid sequence data analyses were performed using the DNASIS program. The homology searches of DNA and amino acid sequences were performed on the BLAST server maintained at the National Center for Biotechnology Information (Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/blast/) using the BLASTP and BLASTX programs. All sequences are available from GenBank, and were aligned using CLUSTAL W software.

Transformation of LAB Plasmid DNA was isolated using the DNA-spin™ Plasmid DNA Purification kit (iNtRON, Seongnam, Korea). *E. coli* was transformed according to the method of Hanahan (22), and LAB were transformed by electroporation as described by Holo and Nes (23) using a Gene Pulser® (Bio-Rad, Hercules, CA, USA), with some modifications. Cells were electroporated in a 0.2-cm cuvette with a Gene Pulser at 25 μF, 200 Ω, and 2.5 kV for *Lb. plantarum* and *Lc. lactis*, and at 25 μF, 400 Ω, and 1.0 kV for *Leu. mesenteroides* and *Lb. paraplantarum.*

Evaluation of plasmid stability The segregational stability of the plasmid was determined using LAB harboring recombinant plasmid pUCL2.1, which carries the erythromycin-resistance gene as a selection marker, as described by Roberts *et al.* (24). A single colony was used to inoculate the selection-pressure-free medium, and the culture was grown at an optimum temperature overnight. The saturated culture was diluted 1,000-fold using fresh medium in the absence of selection pressure. This consecutive transfer of culture into fresh medium was performed every 24 hr at the same dilution ratio (1 transfers is approximately 10 generations). Diluted culture samples were plated as single cells on agar at various intervals and incubated at 37°C overnight. Colonies were plated onto medium containing erythromycin to check for the presence of recombinant LAB.

Results and Discussion

Characterization of pCL2.1 from *Lc. lactis* ssp. *lactis* ML$_8$ According to the previously reported nucleotide sequence of pCL2.1 (Fig. 1A), the amino acid sequence of ORF2 (nucleotides 1657-1938 in pCL2.1) showed 92% identity with the partial C-terminal amino acid sequence of RepB (nucleotides 1917-2198) in pCI411 from *Leu. lactis* 533 (17). In the present sequence reanalysis of pCL2.1, the deduced amino acid sequence upstream of ORF2 (nucleotides 1395-1655 in pCL2.1) showed 77% similarity with the N-terminal amino acid sequence of RepB (nucleotides 1653-1913) in pCI411. Hence, the entire nucleotide sequence of pCL2.1 was reanalyzed, and the sequence in GenBank (U26594) was revised. The revised nucleotide sequence of pCL2.1 was 2,047 bp long (Fig. 1) and contained the entire *repB* gene, the transcriptional repressor protein gene (*copG*), *sso*, and *dso*.

The revised putative RepB of pCL2.1 comprises 203 amino acids and exhibits 88% similarity with RepB of pCI411, and 87% similarity with RepB of pPB1 (Table 1). The revised nucleotide positions from 1129 to 1263 in pCL2.1 were similar to the *repA* in pPB1 and the sequence was deduced as *copG*. The putative CopG protein has 45 amino acids and exhibits 80% similarity with RepA of pPB1, and 75% similarity with CopG of pSSU1 (Table 1, Fig. 2). The overall structure of the Cop protein comprises a conserved helix-turn-helix (HTH) motif and ribbon-helix-helix (RHH) motif, which may be involved in DNA recognition (25,26). The putative CopG protein in pCL2.1 contained the HTH and RHH motifs expected for DNA recognition and repression of the MetJ/Arc subfamily, respectively. Their consensus patterns were highly conserved (Fig. 2) and the Gly residue - which is present in most Cop proteins - was detected. Also, the putative promoter, a –35 region (5'-TTAAAA-3', nucleotides 1065-1069) and an extended consensus –10 region (5'-TGNTATAAT-3', nucleotides 1090-1098), and SD sequence were observed at the upstream of *copG* (Fig. 1).

The Cop protein with antisense RNA [countertranscribed RNA (ctRNA)] is reportedly involved in regulating replication in plasmids (27). ctRNA was observed in the revised pCL2.1, and it contained typical consensus features, a putative promoter, and inverted repeat sequences. Generally, the promoter is found between the complimentary sequence of *copG* and *repB* and the inverted repeat sequences is detected downstream of the putative promoter. The ctRNA of pCL2.1 is encoded at the complementary strand between *copG* and *repB* (nucleotides 1265-1340, Fig. 3A). A putative promoter of ctRNA consisting of a less-conserved –35 region (5'-TAGTTT-3', nucleotides 1340-1335) and an extended consensus –10 region (5'-TGNTATAAT-3', nucleotides 1321-1313) was also observed on the complementary strand. There are inverted repeat sequences at a short distance downstream from this putative promoter. *copG* and *repB* were preceded by potential Shine-Dalgarno (SD) sequences, and have SD/anti-SD binding free energies (ΔG°) of –13.9 and –9.4 kcal/mol, respectively (28). Therefore, these results in pCL2.1 suggest that CopG and ctRNA are involved in the replication of pCL2.1.

The *dso* sequence in pCL2.1 (nucleotides 995-1017) had a high potential to form a stem-loop structure containing

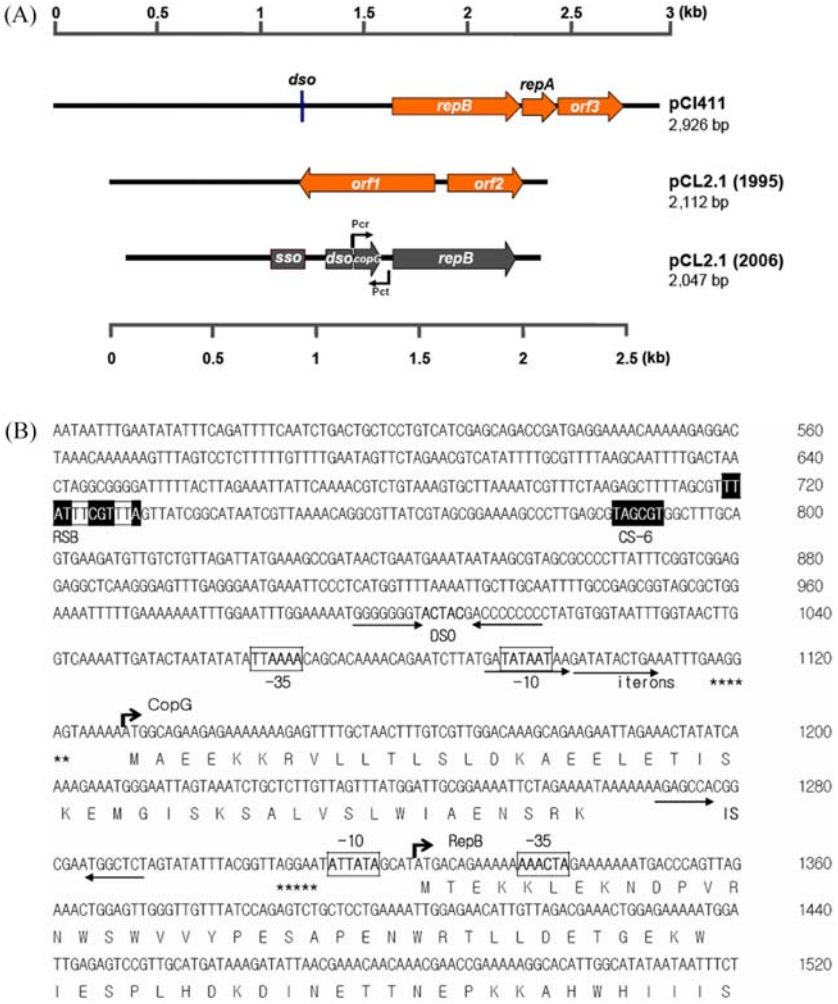

Fig. 1. Gene structure (A) and nucleotide sequence (B) of pCL2.1. The upper and lower scales in panel A correspond to pCI411 and pCL2.1, respectively. The putative recombination site B sequence (RSB) and a 6-bp consensus sequence (CS-6) of the *sso* region (nucleotides 716-883) are shown by the black box. A putative stem–loop structure, with the nick site and iteron of *dso*, is shown in bold lettering and with arrows. Putative –35 and –10 regions of *copG* and ctRNA are located in the box. Inverted repeat sequences are shown by arrows. SD sequences of CopG and RepB are indicated by asterisks.

Table 1. Characteristics of the translated ORFs in pCL2.1

ORF	Nucleotides	Size (amino acids)	Related protein (microorganism)	NCBI accession no.	Identity (%)	Similarity (%)
CopG	1129-1263	45	RepA in pPB1 (*Lb. plantarum* BIFI-38)	YP_138218	48	80
			CopG in pSSU1 (*Streptococcus suis* DAT1)	NP_053057	53	75
			CopG in pW2580 (*S. dysgalactiae* ssp. *equisimilis* W2580)	AAX94281	51	75
			ORF C in pWV01 (*Lc. lactis* Wg2)	NP_053449	40	75
			RepA in pMV158 (*L. agalactiae* BIFI-38)	NC_010096.1	48	75
RepB	1323-1931	203	RepB in pCI411 (*Leu. lactis* 533)	NP_862729	78	88
			RepB in pPB1 (*Lb. plantarum* BIFI-38)	YP_138219	77	87
			RepB in pLA106 (*Lb. acidophilus* TK8912)	NP_862699	48	68
			RepB in pAR141 (*Lc. lactis* ssp. *lactis* M14)	ABB89945	39	61

the nick site that matches a consensus pattern (tACTACgac-x-cccc-x(3)-GTg), which was identified about 110 bp upstream of the *copG* start codon (Fig. 3B). In addition, 2 copies of direct repeats (5'-ATATAATAAG-3'), which is a putative Rep binding site, were identified upstream of the *copG* start codon.

The *sso* functions as the initiation site for DNA synthesis on the lagging strand. This site is normally located outside the minimal replicon, which is sufficient for plus-strand replication and consists of *repB* and *dso*. However, its deletion may cause a reduction in the number of copies, plasmid instability, and the accumulation of ssDNA

Fig. 2. Alignment of CopG with relevant regions of selected reference HTH and RHH motifs. Conserved amino acids of the HTH and RHH motifs are indicated by black and white boxed regions, respectively.

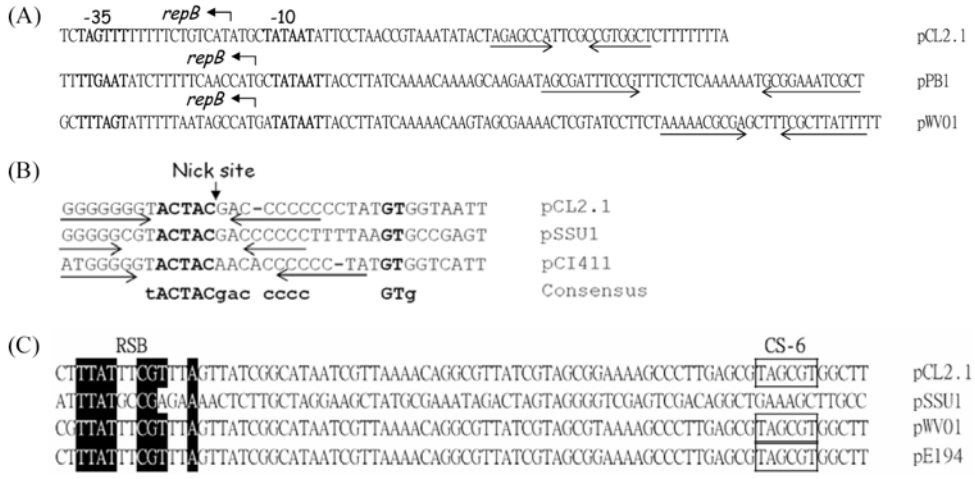

Fig. 3. Comparison of ctRNA, *dso*, and *sso* sequences. (A) Alignment of ctRNAs. Promoter sequences at –35 and –10 are shown in bold lettering. The arrows above the sequences indicate the translation direction of RepB. The arrows below the sequences indicate the inverted repeat sequences. (B) Alignment of the *dso* sequence. The stem-loop structure and nick site are indicated by the horizontal arrows and vertical arrow, respectively. Bold regions represent the consensus sequence of *dso*. (C) Alignment of the *sso* sequence. The conserved regions of the putative RSB and CS-6 sequences are shown by black and white boxed regions, respectively.

intermediates. There are 4 types of *sso* based on similarities in sequence and structure: *ssoA*, *ssoU*, *ssoT*, and *ssoW* (29-33). Typical single-strand origins are palindromic sequences up to 300 bp long and have the potential to form secondary hairpin structures, and they contain 2 conserved sequences: 1) recombination site B (RSB), which is involved in interplasmid recombination (34), and 2) a 6-bp consensus sequence (CS-6, 5'-TAGCGT-3'), which is located within the terminal loop of the major secondary structure of *ssoA* (35). An examination of pCL2.1 for such characteristics revealed a similar sequence at nucleotides 718-728 (5'-TTTATTTCGTT-3'), which was homologous to the RSB of pWV01 (33). The sequence, which had a high potential to form a hairpin structure, was located at nucleotides 716-883 (Fig. 3C). This region also contains the 6-bp consensus sequence 5'-TAGCGT-3' that is present in the loop of single-strand origins (36). Since no other potential *sso* motif was identified on the plasmids, this region may be the *sso* of pCL2.1. The revised pCL2.1 contained the entire *repB*, *copG*, *sso*, and *dso*.

Construction of cloning vector based on pCL2.1 The *E. coli*/LAB shuttle vector pUCL2.1 was constructed to allow evaluation of the replication of pCL2.1 in various LAB (Fig. 4). To implement replication in *E. coli*, pCL2.1 was digested with *Cla*I and ligated into the *Acc*I site of pUC18, and pUCL1.1 was constructed. The fragment containing the erythromycin gene from pIL253 (21) obtained using digestion with *Kpn*I and *Bam*HI was

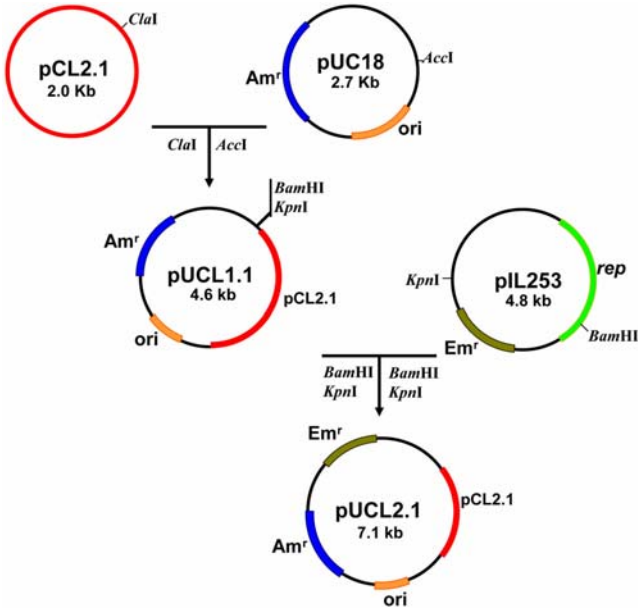

Fig. 4. Construction of the *E. coli*/LAB shuttle vector pUCL2.1. Symbols: ori, replication origin of ColE1; Am^r, ampicillin-resistance gene; Em^r, erythromycin-resistance gene; *rep*, replication protein gene.

introduced into the same site of pUCL1.1, and the resulting plasmid (designated pUCL2.1) comprised the total pCL2.1

Table 2. Transformation efficiencies of various LAB with pUCL2.1, and segregational stability of this plasmid in each host

Strain	Transformation efficiency (CFU/μg DNA)	Segregational stability (%)[1)]	Reference
Lc. lactis MG1363	3×10^3	100	(18)
Lb. paraplantarum C7	1×10^2	100	(19)
Leu. mesenteroides C7	2×10^1	100	(20)
Lb. plantarum KCTC 3109	2×10^2	100	KCTC

[1)]A pUCL2.1 stability of 100% implies the presence of erythromycin-resistant colony-forming units after 40 generations of nonselective growth.

fragment, an *E. coli* replication origin site, an ampicillin-resistance gene for *E. coli*, and an erythromycin-resistance gene for LAB. The LAB listed in Table 2 was successfully transformed by pUCL2.1, with the transformation efficiency varying with the strain under our electroporation conditions.

Segregational and structural stability of pUCL2.1 in LAB The segregational and structural stability of recombinant pUCL2.1 was examined to validate the stability of the pCL2.1 replication system in various LAB. The pUCL2.1 was introduced into *Lc. lactis* MG1363, *Lb. paraplantarum* C7, *Leu. mesenteroides* C7, and *Lb. plantarum* KCTC 3109 (Table 2). The transformation efficiency was lowest in *Leu. mesenteroides* C7, which is a wild-type strain containing several plasmids (data not shown) that could influence the compatibility with pUCL2.1.

There was no pUCL2.1 lost after 40 generations of growth in the absence of selection pressure. Comparison of the plasmid profiles of generation 40 with those extracted from generation 0 by gel electrophoresis revealed that the pUCL2.1 was stably maintained for 40 generations, with no obvious alteration in the size of any plasmids being observed (data not shown).

In conclusion, we have recharacterized the complete DNA sequence of the RCR plasmid pCL2.1, and newly constructed a putative gene organization of pCL2.1 based on the results of homology searching. Plasmid pCL2.1 contains 2 ORFs that encode *copG* and *repB*, a small ctRNA coding region, *dso*, and *sso*. The results suggest that this plasmid replicates via a rolling-circle mechanism. pUCL2.1 based on pCL2.1 was shown to be replicated in various LAB. In addition, recombinant LAB harboring pUCL2.1 were stably maintained under nonselective conditions for 40 generations. Our evaluations of pUCL2.1 in various LAB suggest that this plasmid will be useful for cloning in LAB and will allow the further development of LAB as acceptable hosts for the production of various proteins, peptides, and metabolites. Moreover, pCL2.1 will be useful as a frame for constructing food-grade vectors.

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