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Analysis of nucleotide sequence of a novel plasmid, pILR091, from *Lactobacillus reuteri* L09 isolated from pig

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Abstract : The genus *Lactobacillus* is the largest of the genera included in lactic acid bacteria and is associated with mucosal membranes of human and animal. Only a few *Lactobacillus* plasmid-encoded functions have been discovered and used. In this study, a novel plasmid (pILR091) was isolated from a wild *L. reuteri* isolated from pig and described the characteristics of its replicons, genetic organization, and relationship with other plasmids. After digestion of the plasmid, pILR091, with *Sal*I, plasmid DNA was cloned into the pQE-30Xa vector and sequenced. The complete sequence was confirmed by the sequencing of PCR products and analyzed with the Genbank database. The isolate copy number and stability were determined by quantitative-PCR. The complete sequence of *L. reuteri* contained 7,185 nucleotides with 39% G-C content and one cut site by two enzymes, *Sal*I and *Hin*dIII. The similar *ori* sequence of the pC194- rolling circle replication family (TTTATATTGAT) was located 63 bp upstream of the protein replication sequence, ORF 1. Total of five ORFs was identified and the coding sequence represented 4,966 nucleotides (70.4%). ORF1 of pILR091 had a low similarity with the sequence of pTE44. Other ORFs also showed low homology and *E*-values. The average G-C content of pILR091 was 39%, similar with that of genomic DNA. The copy number of pILR091 was determined at approximately 24 to 25 molecules per genomic DNA. These results suggested that pILR091 might be a good candidate to construct a new vector, which could be used for cloning and expression of foreign genes in *lactobacilli*.

Keywords : G-C content, *Lactobacillus reuteri*, ORF, plasmid DNA, replication

Introduction

The genus *Lactobacillus* is by far the largest of the genera included in lactic acid bacteria (LAB) and is found in very heterogeneous species with a large variety of phenotypic, biochemical, and physiological properties [30, 31]. *Lactobacilli* are widespread in nature. Many *Lactobacilli* species have applications in the food industry and are associated with mucosal membranes, such as the oral cavity, gastrointestinal tract, and vagina of human and animals [6, 15, 33].

Lactobacillus plasmids were first isolated from *L. casei*, and then from a variety of other *lactobacilli* [5]. Generally, *Lactobacillus* spp. have one to ten plasmids, which vary in size from 1.2 to 169 kb [22, 29]. Their functions are divided into four main groups: 1) hydrolysis of proteins; 2) the metabolism of carbohydrates, amino acids, and citrate; 3) the production of bacteriocins, exopolysaccharides, and pigments; and 4) resistance to antibiotics, heavy metals, and phages [3, 37, 39].

Circular bacterial plasmids use two modes of DNA replication, rolling circle (RC) and theta. RC plasmids have been assumed to be the most widespread in gram-positive bacteria. However, in recent years a large number of theta-replication plasmids have been characterized in gram-positive hosts, especially LAB [39]. The RC type of replication seems to be typical for small LAB plasmids (< 12 kb) and is rather unstable [12, 14, 17]. Theta-replication is generally present in medium or large size plasmids and replicates by means of a double-stranded rather than a single-stranded

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replication intermediate, which results in better structural stability [2]. However, the host range of the theta-replication plasmid is rather limited in comparison to RC plasmids.

This growing interest to characterize *Lactobacillus* replicons led to the development of a shuttle vector as a potential useful vector [25]. Kullen and Klaenhammer [20] reported that the plasmid vectors most widely used for *lactobacilli* are three types: plasmids based on rolling circle replication (RCR) replicons, plasmid with two origins of replication (one for *Escherichia coli* and a second for gram-positive bacteria), and *Lactobacillus* vectors with an alternative replication origin for gram-negative bacteria. While 23 plasmids have been sequenced, only a few plasmid-encoded functions have been discovered and applied to vector construction, strain identification, detection, and modification [39]. Furthermore, commercial vector systems were rarely present for the application of *Lactobacilli* to livestock management, compared with the usefulness of probiotic *Lactobacilli* strains.

In this paper, we isolated plasmid DNA from a *L. reuteri* isolated from pig and determined the complete sequence of a new plasmid DNA. We then described the characteristics of its replicons, genetic organization, and their relationship with other plasmids.

Materials and Methods

Bacterial strains and growth conditions for plasmid isolation

Lactobacillus reuteri L09, a wild isolate from pig, was grown in static de Man, Rogosa, and Sharpe (MRS) broth (Merck, USA) at 30°C anaerobically. Plasmid DNA was extracted using the method of O'Sullivan and Klaenhammer [24].

Cloning and analysis of plasmid DNA

Total plasmid DNA was subjected to restriction enzyme analysis with a list of restriction enzymes, including: *Sal*I, *Sph*I, *Eco*RI, *Pst*I, *Xho*I, *Bam*H, *Kpn*I, *Hind*III, *Sma*I, *Xma*I, and *Apa*I (Takara, Japan). The *Sal*I site of the pILR091 was used to produce a linear plasmid DNA. The plasmid was cloned into a pQE30Xa expression vector (Qiagen, The Netherlands) to form pIL-1 at 20°C for 5 h, and then transformed into competent *E. coli* M15 by heat shock method. The transformants were plated on LB agar containing 25 μg/ml of kanamycin and 100 μg/ml of ampicillin, then incubated at 37°C for 16 h. The cloned gene was analyzed by restriction enzyme analysis and sequencing.

Sequencing

The sequence was determined using a ABI 377 automated DNA sequencer (Perkin-Elmer, USA) at the Macrogen (Korea). pQE-universal primers were used to obtain the first sequence and five pairs of walking primers were designed to obtain the complete pILR091 sequence (Table 1). The complete sequence was confirmed by the re-sequencing of PCR products, which was performed using six pairs of walking primers (Table 1). The individual sequence files were edited manually for assembly.

Sequence analysis of pILR091

The pILR091 sequence was analyzed using the Blast programs at NCBI for similarity search, ORF finder for finding ORFs, and DNasist (v1.02; Hugh Patterton, USA) for comparing similarity and chemical and physical properties.

Determination of plasmid copy number

The copy number of plasmid DNA was measured by quantitative-PCR. To calculate the absolute copy number, the concentration of pIL-1, which was a partial pILR091 clone 5.6 kb in size, was measured and the copy number was calculated by the following equation: 1 μg of 1,000 bp plasmid DNA = 9.1×10^{11} molecules [11]. Ten-fold serial dilutions of plasmid solutions were used as a standard sample in each PCR run. Total DNA and standard samples were used as template DNA for PCR after the measurement of concentration. PCR primers were designed to contain the replication region. DNA band density on an agarose gel was analyzed by the Quantity-One program (Bio-Rad, USA) and was used to generate a standard curve and equation. The copy number and amount of plasmid DNA were calculated by the equation of Giulietti A and the standard equation. The amount of genomic DNA was obtained by subtracting plasmid DNA from total DNA [11]. The copy number of genomic DNA was also calculated by the equation of Giulietti A and compared with plasmid DNA [11]. The size of the genomic DNA applied was about 2 Mb because the genomic DNA of *Lactobacillus* spp. was estimated to be between 1.84 and 2.36 Mb [1, 8, 21, 22]. PCR was performed for

Table 1. Nucleotide sequences for sequencing by primer walking and calculating the copy number of pILR091

Purpose	Target	Primers	Direction	Sequence of oligomers	The size of oligomers
Sequencing	pIL-1	pQE primer*	Forward	5'-CCCGAAAAGTGCCACCTG-3'	18
		pQE primer*	Backward	3'-GGTCTTACTGGAGTCTTG-5'	18
		Left	Part #1	ACCATCACCATCACGGAT	20
			Part #2	TCACTTTGGCGTTTAAGTTGG	21
			Part #3	AGACAGCAAGGTCTTGTTTATGAAT	25
			Part #4	CGACCATTTTAGAAAGGAGTAGG	25
			Part #5	TATTATCAGCTTGAACTTGGCTCAT	25
		Right	Part #1	AGTTCCCGCAATGGACTATG	20
			Part #2	GAATCTCAATCTTTGATGCGTTT	25
			Part #3	GACTTCCATACCATGTTTACCAG	25
			Part #4	ATTCTACTGATACCAACCAATTC	25
			Part #5	GTCGAAGCCCTAACTAGATTTGAC	24
	pILR091	Portion #1	Forward	5'-TTATTGTGGTGTTGCTGTAACAG-3'	25
			Backward	3'-AGCTCTCTAGCGTAATTTGAACG-5'	25
		Portion #2	Forward	5'-CGCAAGTTAGGGTTATGAATATCTG-3'	25
			Backward	3'-TCAAAATCATATCGGAATTCTTGTT-5'	25
		Portion #3	Forward	5'-CGCATATTCACATTGTTTTGATTAC-3'	25
			Backward	3'-TTGATCCATTTTTGGGTAGTCTT-5'	25
		Portion #4	Forward	5'-ATCTTGATTGAGTATGGTGTTTGGT-3'	25
			Backward	3'-GTTATCATGTTGGCTTGAATGAA-5'	25
		Portion #5	Forward	5'-TTGATGAGTTCGATTACATTTGA-3'	25
			Backward	3'-CGATAATGTTGACAATTAACAAACG-5'	25
		Portion #6	Forward	5'-CATCTGAAACAAGTCAAAGAGGAAT-3'	25
			Backward	3'-TTACAGGTCCACCATAATAATCACC-5'	25
Copy No.	pILR091	*ori* frag.†	Forward	5'-ACCCTCTGATTCGGTGTTTG-3'	20
			Backward	3'-AGTTCCCGCAATGGACTATG-5'	20

*pQE universal primers. †frag. denotes the fragment of pILR091.

25 reactions with the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec (Table 1).

The stability of plasmid DNA

To test the stability of pILR091, *Lactobacillus reuteri* L09 was grown in 10 ml of MRS broth without antibiotics. Ten μl of a 10^{-3} dilution of the culture was re-inoculated into 10 ml of a fresh MRS every 24 h, which was repeated for approximately 20 generations. The plasmid copy number was then measured as described above.

Results

Plasmid isolation and enzyme digestion

The plasmid, which was named pILR091, was isolated from *Lactobacillus reuteri* L09 and its size was estimated to be about 7 kb by restriction fragment analysis. This was confirmed by the complete sequencing of 7,185 nucleotides. Overall, the G-C content of the plasmid was 39%, while the G content was 1,505 nucleotides (20.9%), T was 2,063 nucleotides (28.7%), A was 2,319 nucleotides (32.3%), and C was 1,298 nucleotides (18.1%). The sequences were submitted to GenBank database and received accession number, EF432638.

Total plasmid DNA of *Lactobacillus reuteri* L09 was digested with several restriction enzymes, and it was identified that two enzymes, *Sal*I and *Hin*dIII, had only one-cut site. Furthermore, it showed that *L. reuteri* L09 had two plasmid DNAs less than 10 kb of size. The *Sal*I site was used to clone pILR091 into *E. coli* vector pQE30Xa, and the enzyme site was confirmed by sequence analysis (data not shown).

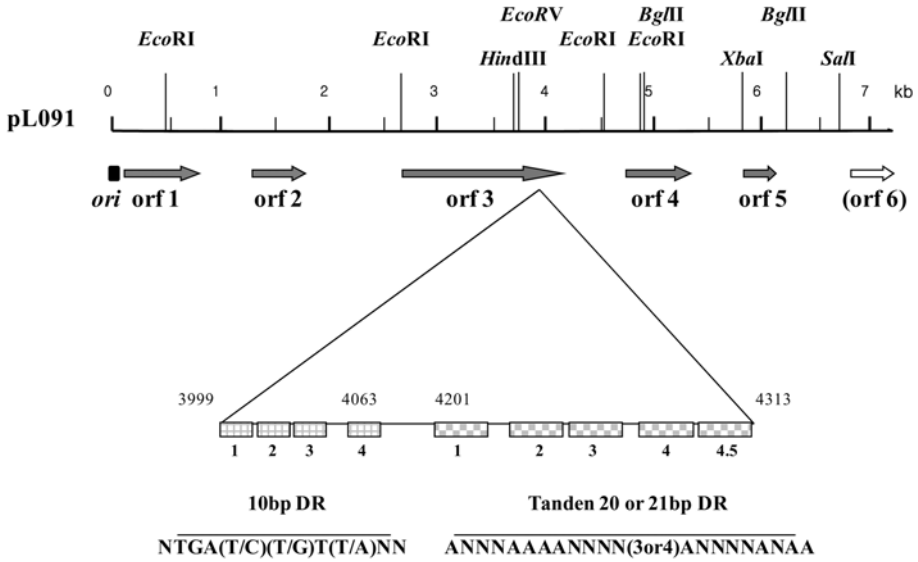

Fig. 1. Structural organization of the putative ORF, replication region of pILR091, and restriction enzyme. The ORFs are shown by black (replication protein) and gray (putative protein) arrows, and the vestige of a putative ORF by an empty arrow. The replication origin is indicated by the black square. Gray lattices and checks denote a 10 bp DR and 21 or 22 bp DR, respectively.

pC194-RCR family	1	TCTTATCTTGATAC	14
L. plantarum pLTK2	1	TCTTATCTTGATA	13
L. casei pLC88	1	TTCTTCTTATCTTGAT	16
L. reuteri pGT232	1	CTTATCTTGATA	12
L. reuteri pTE44	1	CTTATCTTGATA	12
L. reuteri pILR091	1	GGCCGTGTGGTTTATATTGATAACACTCGGATTGCACA	38

Fig. 2. Comparison of *ori* sequence between *Lactobacillus* spp.. The replication origin sequence was aligned with those of *Lactobacillus* spp. using the DNasist program. The filled square indicates the conserved region between *ori* sites, and the putative replication origin of pILR091 is indicated by an empty square.

Identification and comparative analysis of the putative replication region

The consensus nick-site sequence of the reported RCR was applied to the pILR091sequence. A consensus sequence of the pC194-RCR family (TCTTATATCTT GATAC) and the *ori* site of a cryptic plasmid from *L. curvatus* LTH (TACTACGA) were found in the sequence of pILR091 (Fig. 1). The sequence of pC194-RCR was located 63 bp upstream of the protein replication sequence, ORF 1, and may be shows according to belongs a high similarity with other *ori* sites of *Lactobacilli* (Fig. 2). Furthermore, the *ori* site of *L. curvatus* [18] was located in the ORF 3 sequence site and had no near ORF sequence that was recognized as the replication protein (Fig. 1). The putative replication origin of pILR091 (TTTATATTGAT) belongs to the pC194-RCR family, but showed some sequence variation in this study.

Two types of repeat sequences were found downstream of ORF 3 (Fig. 1). The first of these repeat sequences is a 10 bp direct repeat (NTGA(T/C)(T/G)T(T/A)NN), which repeats four times, and the second is a 20 or 21 bp putative iteron [ANNNAAAA NNNN(3 or 4)ANNNNANAA], tandem repeated 4.5 times.

Table 2. General features of putative ORFs from pILR091 with best match to public database sequences

ORF	Position (bp)	Size (aa)	Proposed function	Source strain	E-value	amino acid identity (%)	Genbank Accession No.
1	75-869	264	Replication initiation protein	*Lb. reuteri*	9e-09	24	AAF18392.1
2	1338-1847	169	Hemolysin	*Fu. nucleatum subsp. vincentii ATCC 49256*	0.014	25	ZP_00144284.1
3	2676-4166	496	hypothetical protein Lreu23DRAFT_0454	*Lb. reuteri*	3e-46	32	ZP_01275098.1
4	4710-5396	228	hypothetical protein PFL0045c	*Pl. falciparum 3D7*	1.2	25	NP_701374.1
5	5591-6137	48	unknown extracellular protein lr1990	*Lb. reuteri*	7e-20	100	AAY86924.1
(6)	6891-?	98	101aa long hypothetical protein	*Py. horikoshii OT3*	0.26	30	BAA30779.1

Fig. 3. Comparison of the replication proteins from *L. reuteri* pILR091 and pTE44. The pILR091 replication protein sequence was aligned with pTE44, which is another plasmid DNA from *L. reuteri* using the DNasist program. The bold and larger letters indicate conserved amino acid sequences.

General feature of putative ORFs

A total of five ORFs were identified (Fig. 1) and the coding sequences represent a total of 4,966 nucleotides, which occupied 70.4% of the plasmid. A vestige of one-putative ORF was also found as expected (Table 2).

Putative replication protein

ORF1 of pILR091 has been identified as responsible for plasmid DNA replication and has displayed identities with amino acid sequences of several gram positive bacteria besides *Lactobacillus reuteri*, such as *Oenococcus oeni* (Genbank Access. No. BAD91409.1, *E*-value 4e-09, 24% identity; No. NP_254271.1, *E*-value 9e-09, 22% identity, respectively). Even though low *E*-values were observed, *Staphylococcus aureus* also showed a significant identity (No. YP_492679, *E*-value, 0.18, 21% identity; No. AAM94141.1, *E*-value 0.14, 22% identity; No. CAD55143.1, *E*-value 0.31, 21% identity). ORF1 of pILR091 had the lowest similarity with the pTE44 protein sequence, which is another plasmid DNA from *L. reuteri* (Fig. 3). Other replication proteins of *Lactobacillus* spp. also showed low homology values.

Putative ORFs

OFR 2 showed similarity with hemolysin of *Fusobacterium nucleatum* subsp. *vincentii* ATCC 49256, but also with several Knob-associated histidine-rich protein precursors despite having a low *E*-value. ORFs 3 and 4 were not identified. ORF 5 showed a high amino acid identity (100%) and *E*-value (7e-20), but its function was not clearly defined. ORF 6 might be a vestige ORF because it showed identity with several amino acids in a 101 aa hypothetical protein, 2-oxoglutarate dehydrogenase E1 component, heat shock protein 16.9, and a porin, but lacked a genetic code terminator such as TAA, TGA, and TAG (Table 2).

The identity and function of ORFs were searched using the Blast program and the protein database. But the identity, function, and *E*-value were generally low except for the ORF from *L. reuteri*.

Table 3. Comparison of G-C content between cryptic plasmid and non-cryptic plasmid DNA

Plasmid				*Lactobacillus* strains		
Name		G-C content (%)	Size (bp)	Strains	G-C content (%)	References
Cryptic (RC-replication)	pC30i1	37	2,140	*L. plantarum*	44.5	(34)
	pKC5b	33.47	4,390	*L. fermentum*	53 (Bergey's manual)	(25)
	pC7	38.5	2,134	*L. paraplantarum*	44.5 (*L. plantarum*)	(26)
	pWCFS101	39.5	1,917	*L. plantarum*	44.5	(38)
	pWCFS102	34.3	2,365			
Non-cryptic (θ-replication)	pCD1	39.04	19,882	*L. paracasei*	46.6 (*L. casei*)	(7)
	pCD2	39.04	8,554			
	pRV500	38.1	12,959	*L.sakei*	38.1	(2)
	pWCFS103	40.8	36,069	*L. plantarum*	44.5	(38)

Table 4. Comparison of G-C content between ORFs in pILR091 and genomic DNA in *Lactobacillus* strains

pILR091		*Lactobacillus* strains			
ORF	G-C content (%)	Strains	G-C content (%)	GeneBank Accession No.	Genome sequencing
1	38.86	*L. reuteri* JCM1112	38.8	AAOV00000000	In progress
2	38.82				
3	42.75	*L. plantarum*WCFS1	44.5	AL935263	completed
4	32.75	L. gasseri	31.1	AAAO00000000	In progress
5	52.38	*L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365	49.9	AAGQ00000000	In progress
(6)	35.8	*Lactococcus lactis* I11403	35.3	AE005176	completed
		L. acidophilus NCFM	34.7	CP000033	completed
		L. johnsonii NCC533	34.6	AE017198	completed

Copy number and stability of pILR091

The densities of DNA on agarose were analyzed by the Quantity One program and compared with the copy number of plasmid and genomic DNA. The DNA density was equal between 1 ng of pIL-1 (9.1 kb) and 10 ng of genomic DNA (2 Mb). Therefore, the amount of genomic DNA produced was 9 ng. The copy number using the equation of Giulietti A was determined with the following relation, 1 ng of pIL-1 had 1×10^8 molecules and 9 ng of genomic DNA had 4.095×10^6 molecules [11]. The ratio was approximately 1 : 24.5 between genomic and plasmid DNA, indicating that the copy number of pILR091 was between 24 and 25. The copy number kept increasing for 20 generations, which showed that the pILR091 replicated stably in its host, *L. reuteri* L09.

Discussion

The sequencing of pILR091 was completed and it revealed that the overall G-C content of the plasmid was 39% and in accordance with that of a general *Lactobacillus* spp. plasmid [39]. After enzyme digestion with *Sal*I and *Hin*dIII, one cut site was discovered in pILR091, which showed that *L. reuteri* L09 had two plasmid DNAs less than 10 kb in size considering the structural configuration of plasmid DNA [28].

The majority of LAB-associated plasmids replicate by two basic mechanisms, the RCR or theta replication [7]. pILR091 consisted of two putative RCR-replication origins, one putative replication protein, and two types of repeated sequences. The replication origin of pILR091 was compared with that of other plasmid DNA of *Lactobacillus* spp. and the pC194-RCR family. The 11 bp *ori* consensus sequence (5'-CTTATCTTGAT-3') was found in several plasmids, but pILR091 showed little similarity with others [7]. The putative *ori* of pILR091 consisted of 12 bp (5'-TTTATATTGATA-3').

L. curvatus LTH (TACTACGA) was also found in

pILR091 sequences. Many large plasmids, such as those of the IncF incompatibility groups, contain multiple replicons, although only one is active *in vivo* [36]. However, the incompatibility group was located in ORF3 and far from the putative replication protein. Therefore, it might be matched accidentally or inactive replication origin.

Repeated structural configurations is a typical feature of theta replication but protein replication did not occur at both sides of the AT-rich repeat sequence [2, 4, 7, 9, 25]. Theta replication was generally present in medium to large size plasmids, spanning a few thousand to tens of thousands of bp for large plasmids [31]. pILR091 was a relatively small theta-replication plasmid, even though pWV02 was an especially small (3.8 kb) plasmid and pILR091 was a little large compared with other plasmids that use RCR [16].

The replicated protein showed a very low homology to other proteins, even when compared to pET44 from *L. reuteri*. Generally, lactic acid bacteria used as probiotics resulting from different milieux have different characteristics [13]. In particular, *L. reuteri* strains isolated from different hosts have distinctive colony morphologies, while retaining similar physiological and genetic characteristics [30]. This might indicate an adaptation of its surface properties to meet specific host colonization site requirements and demonstrates that plasmid DNA might play a part in their different characteristics. For this reason, *L. reuteri* L09, which was the host of pILR091, had the same physiological and genetic characteristics of *L. reuteri,* but unique characteristics might be reflected by the difference of plasmids, such as pILR091.

The average G-C content of pILR091 was 39% and was similar to that of genomic DNA. The heterogeneity of *Lactobacillus* spp. was reflected by the range of 32-55% G-C content between species, which also applied to their plasmid DNA [32]. These characteristics demonstrated that pILR091 might be a non-cryptic plasmid and have both the advantages of RCR-replication, such as broad host range, and theta-replication, such as stability.

However, each ORF showed a different G-C content that was coincident with the variation of G-C content of *Lactobacillus* spp.. pRV500 from *Lactobacillus sakei* also showed a similar variation of G-C content to that of pILR091 [2]. Furthermore, ORFs 7, 8, 9, 10, 11, and 12 of pRV500 had an identity above 85% with those of other *Lactobacillus* spp., but showed a different G-C content with their hosts. It was difficult to conclude that most ORFs in pILR091 originated from a plasmid stemming from another *Lactobacillus* spp. or gram-positive bacteria, though this origin was not impossible. However, it may be possible that plasmid DNAs, which had a similar G-C content to that of their host, tended to have safe inheritance and adjusted their G-C content to the host by active or passive control, such as rearrangement of genes after transduction or conjugation [10, 36]. The remnant of this adjustment might be the *ori* site of the cryptic plasmid from *L. curvatus* LTH (TACTACGA), the two types of repeat sequence in ORF 3, and the vestige of one-putative ORF 6.

Three different vectors, which were constructed with three different replicons in the same backbone as MCS and selection markers, showed similar stability after 110 generations [38]. This demonstrated that G-C content plays as important a role in stability as replication mode does. In view of this finding, G-C content must be considered and adjusted in the cloning of a target gene, besides only vector construction.

24 to 25 molecules of pILR091 were present per *L. reuteri* L09. The copy number of *L. reuteri* L09 was not yet reported, but other LAB generally had plasmids which replicated between 2 to 15 copy numbers [17, 19, 23, 35]. However, some constructed plasmids have demonstrated 30 to 170 copy numbers [27]. A number of different factors may affect these differences, such as the nature of the host, interactions between the plasmid-encoded elements and the host transcription and replication machineries, and the size and nature of the inserted sequences [17]. Therefore, the copy number of pILR091 also may be changed during vector construction, even though the copy number of pILR091 itself was not changed from its normal state throughout 20 generations.

pILR091 had the same G-C content of genomic DNA which compensated for the disadvantage of being an RCR plasmid. As a result, pILR091 had both the advantages of RCR and theta-replication plasmids. Since the copy number and stability of a plasmid might be related within a given host, *L. reuteri* L09 and this association should be considered to construct a vector for the development of an oral delivery system. Furthermore, several other factors must be considered in order to construct an oral delivery system vector,

such as plasmid stability, the food-grade selection marker, the insertion position of a multicloning site, the display motif, the promoter, and the terminator.

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