Sequencing and analysis of three plasmids from *Lactobacillus casei* TISTR1341 and development of plasmid-derived *Escherichia coli-L. casei* shuttle vectors

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Short title: *Lactobacillus casei* plasmids and plasmid-derived vectors

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Abstract

Pyrosequencing followed by conventional PCR and sequencing was used to determine the complete nucleotide sequence of three plasmids (pRCEID2.9, pRCEID3.2 and pRCEID13.9) from the *Lactobacillus casei* strain TISTR1341. The plasmid sequences were found to be almost identical, respectively, to those of pLA106, pLA105 and pLA103 from *Lactobacillus acidophilus* strain TK8912, suggesting these strains may be related. Sequence analysis and comparison indicated that pRCEID2.9 replicates by a rolling circle (RC) mechanism, while pRCEID3.2 and pRCEID13.9 probably follow a theta-type mode of replication. Replicons of pRCEID2.9 and pRCEID13.9 were used to develop *Escherichia coli*/*L. casei* compatible shuttle vectors, which were stably maintained in different genetic backgrounds. Real-time quantitative PCR analysis showed copy numbers of around 4 and 15 for the pRCEID13.9- and pRCEID2.9-derived shuttle vectors per chromosome equivalent, respectively. Functionality of the pRCEID-LC13.9 vector was proved by cloning and expressing in *L. casei* of a green fluorescent protein gene variant from *Aequorea victoria.* The new vectors might complement those currently in use for the exploitation of *L. casei* as a cellular factory and in other biotechnological applications.

Key words: *Lactobacillus casei*, plasmid, cloning vectors, lactic acid bacteria

1. Introduction

Lactobacillus casei is a member of the lactic acid bacteria (LAB) present in many environments, including milk and dairy products, meat, plant materials and the mucosa of animals and humans (Hammes and Hertel, 2006). Some strains of *L. casei* have been shown to enhance the immune response (Kim et al. 2006) and are used as probiotics. Immunization

with recombinant *L. casei* cells expressing reactive antigens has been found to elicit an immune response (Lee et al. 2006a). Indeed, *L. casei* has been engineered to express a variety of heterologous proteins of bacterial (Maassen, 1999), viral (Ho et al. 2005) and eukaryotic origin (Yao et al. 2004) with vaccination purposes in mind. Expression systems for *L. casei* are usually based on replicons from indigenous cryptic plasmids. As vector amplification and DNA purification are easier to perform in *Escherichia coli* than in *Lactobacillus* species, *E. coli/Lactobacillus* shuttle vectors are generally preferred. Homologous and heterologous protein expression is affected by plasmid properties such as mode of replication, copy number and stability; therefore, the study of the basic biology of plasmids is considered a key factor for expression maximization (Shareck et al. 2004).

To date, 72 plasmids from *Lactobacillus* species have been sequenced, of which 12 (http://www.ncbi.nlm.nih.gov/genome/) are from species of the *L. casei* group (*L. casei, Lactobacillus paracasei, Lactobacillus rhamnosus*, and *Lactobacillus zeae;* Felis and Dellaglio, 2007). Sequenced plasmids range in size from the 3178 bp of pTXW from *L. paracasei* TXW to the 64,508 nt of pLC1 from *L. rhamnosus* Lc 705. Beyond their replication, mobilization and partitioning modules, most plasmids are cryptic. Nevertheless, β-galactosidases, phospho-β-galactosidases and components of PTS systems are encoded in some of the largest. In addition, bacteriocin production and immunity genes have been identified in pLA103 (Kanatani et al. 1995a) and pSJ2-8 (Kojic et al. 2010). Nucleotide sequence analysis supports rolling circle (RC) replication for pSMA23, although evidence of intermediary single-stranded DNA has been obtained only for pTXW (Zhang et al. 2010). The sequence homology and size of the other plasmids above suggest that most replicate by the theta mechanism. Both RC and theta replicons have been used for the construction of cloning vectors, (An and Miyamoto, 2006; Zhang et al. 2010) and in homologous and heterologous

gene expression in lactobacilli (Sudhamani et al. 2008). Cloning vectors based on RC replicons usually have a wider host range than those based on theta-replicating plasmids. Conversely, RC plasmids (and vectors) frequently show greater structural and segregational instability than theta plasmids. Thus, the selection of RC or theta plasmids for vector construction greatly depends on the application.

In the present work, three plasmids from the *L. casei* TISTR1341 strain were completely sequenced and analyzed. Based on the similarity of replication-initiation proteins and the nucleotide sequence of their putative origin of replication, pRCEID2.9 is predicted to be an RC-replicating plasmid, while pRCEID3.2 and pRCEID13.9 are probably theta-replicating. *Lactobacillus/E. coli* shuttle vectors were generated based on the replicons of pRCEID2.9 and pRCEID13.9.

2. Materials and Methods

2.1. Bacterial strains, cloning vectors, primers and growth conditions

Table 1 lists all the bacterial strains, cloning vectors and primers used in this study. *Lactobacillus* strains were cultured statically in MRS medium (Difco, East Molesey, UK) at 37°C. *E. coli* was grown in 2×TY or LB broth at 37°C with shaking. *Bifidobacterium longum* was grown in MRS supplemented with 0.5 % (w/v) cysteine under anaerobic conditions at 37ºC. Agarified media were obtained by the addition of 15 g/l agar to the corresponding broth. When needed, antibiotics were added to the media as follows; for *E. coli*, ampicillin (100 µg/ml) and tetracycline (10 µg/ml); for *L. casei*, erythromycin (2.5 µg/ml) and tetracycline (10 µg/ml).

97 2.2. Plasmid isolation

98 Plasmids from *L. casei* were purified as described by O'Sullivan and Klaenhammer
99 (1993). Plasmid DNA from *E. coli* cells was isolated by the alkaline lysis method as described
100 by Sambrook and Russell (2001). When required, plasmid DNA was purified using a
101 QIAprep® Spin Miniprep kit (Qiagen, Valencia, CA, USA).

102

103 2.3. Curing of native plasmids of *L. casei* TISTR1341

104 Successive elimination of plasmids from the original isolate *L. casei* TISTR1341 was
105 performed by growing the bacteria at 40°C and sub-culturing at this temperature every 24 h in
106 MRS broth. After seven passages, the cultures were serially diluted and plated onto MRS
107 agar. These plates were then incubated at 37ºC for 48 h; colonies were picked at random and
108 analyzed for plasmid content as described above.

109

110 2.4. Carbohydrate fermentation test

111 The sugar utilization capacity of wild type and plasmid-cured derivatives was analyzed
112 using the API 50 CHL system (bioMérieux, Marcy l'Etoile, France).

113

114 2.5. Bacteriocin production assay

115 Twenty milliliters of MRS agar medium at 45°C were vigorously mixed with 200 µl of
116 an overnight culture of the indicator strains *L. sakei* CECT906, *L. casei* ATCC393, and *L.*
117 *rhamnosus* GG, and poured into Petri dishes. Wells were made in the agar layer and 50 µl of
118 filter-sterilized, neutralized (pH 6.5) supernatants from overnight cultures of the strains under
119 assay were added to the wells. Bacteriocin production was scored as a growth inhibition halo
120 around the wells.

121

122 2.6. Molecular DNA techniques

123 General procedures for DNA manipulation were followed essentially as described by
124 Sambrook and Russell (2001). Total genomic DNA from *L. casei* and *B. longum* was purified
125 from overnight cultures using the GenElute^{TM} Bacterial Genomic DNA kit (Sigma; Sigma-
126 Aldrich, St. Louis, MO, USA). PCR products were purified using GenElute^{TM} PCR Clean-Up
127 columns (Sigma). DNA from agarose gels was purified using the GFX PCR DNA Gel Band
128 Purification kit (GE Healthcare Biosciences, Buckinghamshire, UK). Restriction
129 endonucleases (Takara; Otsu, Shiga, Japan), T4 DNA ligase (Invitrogen, Carlsbad, CA) and
130 *Taq* DNA polymerase (Ampliqon, Skovlunde, Denmark) were used as recommended by their
131 manufacturers. White/blue screening was performed for *E. coli* XL1-Blue on LB plates
132 supplemented with the appropriate antibiotic, 5-bromo-4-chloro-3-indolyl-ß-D-
133 galactopyronoside (20 mg/ml) (X-Gal; Sigma) and isopropyl-ß-D-thiogalactopyranoside (0.5
134 M) (IPTG; Sigma).

135 The molecular identification of *L. casei* TISTR1341 was performed by partial
136 amplification of its 16S rRNA genes with the universal prokaryotic primer pairs 27F and
137 1492R (Table 1), sequencing, and comparison against sequences held in databases.

138

139 2.7. Plasmid sequencing, assembly and analysis

140 The complete nucleotide sequence of three plasmids from *L. casei* TISTR1341 was
141 determined using the 454 Life Sciences Genome Sequencer (GS) FLX platform (Roche;
142 Roche Applied Science, Indianapolis, IN, USA) at the in-house facility of the National Center
143 for Genetic Engineering and Biotechnology, Thailand. The nucleotide sequence reads
144 obtained were assembled using Newbler *de novo* sequence assembly software (Roche). Gaps

were filled by DNA amplification using conventional PCR techniques, cloning and sequencing. The primer pairs used in these amplifications are summarized in Table 1. Amplicons were cloned into the pGEM-T Easy cloning vector (Promega, Madison, USA) and sequenced with a MegaBACE 1000 sequencer (BioDesign Co. Ltd., Pathumthani, Thailand) using M13 forward and reverse primers.

Plasmid sequences were analyzed and compared using CLC workbench 5.6 software (http://www.clcbio.com/index.php?id=92). Open reading frames (ORFs) were analyzed on both DNA strands using DNAMAN software (Lynnon Corporation, Pointe-Claire, Quebec, Canada). Putative promoter and ribosome binding site (RBS) sequences were searched for by comparison with consensus sequences (TTGACA for -35 box, TATAAT for -10 box and AGGAGG for RBS). Direct (DR) and inverse (IR) repeats, restriction endonuclease sites and plasmid maps were determined using Clone Manager 7.0 software (Scientific and Educational Software, Cary, NC, USA). Sequence similarity searches were performed using the NCBI database BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Protein structure and motives were searched using the pfam search tool at (http://pfam.sanger.ac.uk/).

2.8. Electrotransformation

Electrotransformation (electroporation) of *E. coli* was performed using a Gene Pulser apparatus (Bio-Rad, Richmond, CA, USA) as described by Dower et al. (1998). Preparation of competent cells and electroporation of *L. casei* was performed as previously described by Chassy and Flickinger (1987).

2.9. Segregational and structural stability of the constructs

168 The segregational and structural stability of the constructs was studied in *L. casei*
169 RCEID02, a plasmid-free derivative of TISTR1341. For segregational stability studies,
170 transformants carrying vectors pRCEID-LC2.9 or pRCEID-LC13.9 were grown in MRS broth
171 without antibiotics for approximately 100 generations. Every 20 generations an aliquot of the
172 culture was removed, diluted and plated onto antibiotic-free medium. Colonies were then
173 replicated on media with and without antibiotics. Plasmid structural stability was checked by
174 restriction analysis of plasmids isolated from the colonies grown.
175
176 2.10. Determination of plasmid copy number
177 Real-time quantitative PCR (Q-PCR) was used to determine the relative copy number of
178 two shuttle vectors pRCEID-LC2.9 and pRCEID-LC13.9 in *L. casei* RCEID02, essentially as
179 described by Lee et al. (2006b). The copy number of the constructs was calculated using the
180 formula $N_{relative} = (1+E)^{-\Delta C}{}_T$ (Lee et al. 2006b), where *E* is the amplification efficiency of the
181 target and reference genes, and ΔC_T the difference between the threshold cycle number (C_T)
182 of the reference gene and that of the target. DNA amplification and detection was performed
183 in a Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR®
184 Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). *repB* and *repA1* were
185 used as the targets for copy number quantification of pRCEID-LC2.9 and pRCEID-LC13.9
186 respectively. The transcription elongation factor encoded by the *greA* gene, a single-copy,
187 chromosomally-encoded gene on the genome of *L. casei* BL23 (Mazé et al. 2010), was used
188 as a reference. Primers were designed using LightCycler Probe Design Software 2.0 (Roche)
189 (Table 1). A 123 bp segment of the *repB* gene of pRCEID-LC2.9 was amplified with the
190 primer pair RepB2.9-F1 and RepB2.9-R1. RepA13.9-F1 and RepA13.9-R1 primers were used
191 to amplify a 129 bp segment of the pRCEID-LC13.9 *repA1* gene. As a control, a 122 bp

segment of the *greA* gene was amplified with primers TEF-F1 and TEF-R1. Q-PCR reactions were all performed in duplicate.

2.11. Nucleotide sequence accession number

The complete nucleotide sequences of pRCEID2.9, pRCEID3.2 and pRCEID13.9 were deposited in the GenBank database under accession numbers HQ173810, HQ259051 and HQ259052, respectively.

3. Results and Discussion

3.1. Molecular identification of the TISTR1341 strain

L. casei TISTR1341 had been isolated from the intestine of a healthy chicken and shown to possess good probiotic properties for chicken rearing (Thanaruttikannont, 1996). Originally, it was identified as *L. casei* by phenotypic profiling via carbohydrate utilization, a method repeatedly reported to be unreliable for lactobacilli (Felis and Dellaglio, 2007). To assure the identity of strain TISTR1341, 16S rRNA gene amplification and sequencing was performed. Sequence analysis and comparison showed 100% identity with 16S rDNA from several *L. casei* strains in databases, including those from strains BL23 (HM162415) and Zhang (CP001084). This result confirmed that TISTR1341 belonged to the *L. casei* species.

3.2. Plasmid content of *L. casei* TISTR1341

Using agarose gel electrophoresis, the plasmid profile of *L. casei* TISTR1341 was shown to be consistently composed of five DNA bands of approximate molecular masses of 3, 3.2, 7.5, 14 and ~30 kbp (Figure 1). These bands may represent different plasmids; although some

could be different forms (super coiled, circular or linear) of the same molecule, as was the case for a weak band seen at the 5.0 kbp position. The biological significance of plasmids in lactobacilli species remains unknown. Some species harbor a vast array of distinct molecules, while others are mostly plasmid-free (Wang and Lee, 1997). Permissible species carry an abundant plasmid complement, composed of both cryptic and phenotype-encoding molecules. The latter plasmids are used to encode adaptative traits, some of which are critical for their industrial applications (Shimizu-Kadota, 1987; Chassy and Alpert, 1989). Plasmid replicons are also essential for the development of genetic tools useful in molecular studies, and for genetic engineering of *Lactobacillus* and other LAB species (Shareck et al. 2004).

After curing, a series of strains having lost different plasmid combinations of the original TISTR1341 complement was obtained, including an isolate that proved to be plasmid-free (RCEID02). The wild type and some plasmid-cured derivatives were examined for carbohydrate utilization and bacteriocin production, two phenotypic traits typically encoded by plasmids in *L. casei* (Chassy and Alpert, 1989; Zhang et al. 2008; Kojic et al. 2010). These analyses associated plasmid pRCEID13.9 with bacteriocin production and immunity. Neutralized supernatants of the wild type strain TISTR1341 were shown to inhibit several *Lactobacillus* spp. indicators, including *L. casei* ATCC393, *L. rhamnosus* GG, and *L. sakei* CECT906. However, this inhibition ability was lost when pRCEID13.9 was absent. In addition, pRCEID13.9-cured derivatives were found to be susceptible to the bacteriocin produced by the original isolate. Plasmid pRCEID30 seemed to be involved in sugar utilization, as derivatives losing this plasmid and the plasmid-free strain RCEID02 showed a reduced carbohydrate fermentation profile after 48 h of incubation (data not shown). However, utilization of 26 carbohydrate substrates of the API 50 CH system was identical after 7 days of incubation. Therefore, essential components are not encoded in pRCEID30.

240

241 3.3. Sequencing, sequence comparison, and plasmid assemblage

242 To determine the sequence of plasmids from TISTR1341, purified plasmid DNA from
243 this strain was subjected to pyrosequencing analysis. Initially, pyrosequencing reads of an
244 average length of 150 bp were assembled into 2314 contigs, ranging in size from 99 to 17308
245 bp. Two contigs (0057, 13510 bp; 02265, 426 bp) could be assembled into a circular molecule
246 of 13908 bp, which corresponded to the whole molecule of pRCEID13.9. This was proved by
247 PCR amplification and sequencing of the overlapping regions. The DNA sequence of
248 Prceid13.9 showed a high nucleotide identity to the entire molecule of plasmid pLA103 from
249 *Lactobacillus acidophilus* TK8912 (AB081463). The nucleotide sequence of three contigs
250 (02294, 1273 bp; 02295, 1367 bp; 02285, 906 bp) showed strong similarity to parts of
251 plasmid pLA105 (D49554) from the same *L. acidophilus* strain. However, compared to
252 pLA105, two gaps were found, suggesting that the plasmid sequence was not complete.
253 Similarly, contig 00205 (2948 bp) was very similar to a major part of plasmid pLA106
254 (D88438), with a short gap of only 4 bp. To bridge the gaps between contigs, PCR was
255 performed with primers designed on the sequences on either side of the gaps. Amplicons were
256 then cloned and sequenced, and the sequences assembled with the existing contigs. As a
257 result, the sequences of plasmids pRCEID2.9 (2952 bp) and pRCEID3.2 (3250 bp) were also
258 completed.

259 Long stretches of the largest contig showed a high nucleotide identity to sequences of *L.*
260 *casei* bacteriophage A2 (AJ251789.2), and to phage-related sequences from the whole
261 genome of *L. rhamnosus* Lc 705 (FM179323.1). Sequences highly similar to those of pCD01
262 from *L. paracasei* NFBC338 (Desmond et al., 2005) and to other theta-type plasmids from *L.*
263 *casei* and *L. rhamnosus* strains were also observed in contig 2310 (6814 bp). These sequences

may be related to either pRCEID30 or pRCEID7.5. However, sequences were not unequivocally identified; therefore, their analysis was not included.

3.4. Analysis of pRCEID2.9, pRCEID3.2 and pRCEID13.9

The nucleotide sequence of plasmids pRCEID2.9, pRCEID3.2 and pRCEID13.9 have a G+C content of 43%, 44.3% and 41% respectively, percentages that agree well with the G+C content of other lactobacilli plasmids, and just a little lower than that of the *L. casei* chromosome (46%; NC_008526). Plasmid sequences were analyzed for translated (open reading frame, ORF) and untranslated sequences (direct, inverse and mirror repeats). Deduced proteins from all ORFs were compared to those in protein sequence databases. Based on protein similarity, pRCEID2.9 and pRCEID3.2 seem to encode only proteins involved in replication and mobilization, while pRCEID13.9 is likely to be organized into two major functional regions, one including the replication module and the other involved in bacteriocin production, immunity and export. The ORFs identified in each plasmid together with the deduced proteins and the proteins in databases to which they showed the maximum homology are summarized in Table 2.

The nucleotide sequence of pRCEID2.9 was 99% identical to that of pLA106 from *L. acidophilus* TK8912 (D88438; Kanatani et al. 1995a). The differences included a single nucleotide insertion at position 67, single nucleotide deletions at positions 1735 and 1803, and a double insertion at position 1779 (all located in non-coding regions). However the most striking difference between the two plasmids was an 83 bp insertion in pRCEID2.9 embraced by two *Pst*I sites (of which only one is present in pLA106) at position 1487. This insertion was located at the terminal part of the *mobA* gene, see below. Altogether, these changes make the sequence of pRCEID2.9 90 bp longer than that of pLA106. Sequence analysis revealed

pRCEID2.9 to consist of three ORFs. ORF1 (*mobA* gene) encodes a 436 amino acid protein with a Mob_Pre domain (pfam01076) that shares 99% amino acid sequence similarity with the mobilization proteins of plasmids pTXW (ADD64221.1) and pLA106 from *L. paracasei*, and *L. acidophilus* respectively. However, a consensus *oriT* region upstream of its putative start codon was not identified. ORF2 encoded 51 amino acids, showing 100% similarity to the repressor protein (RepA) of plasmid pLA106. This plasmid has been shown to belong to the pMV158 RC-replicating plasmid family (del Solar et al., 1998). Finally, the gene product of ORF3 has an encoding capacity of 193 amino acids, which showed 100% similarity to the replication initiation protein RepB of plasmid pLA106 from *L. acidophilus* (Sano et al. 1997), and 61% similarity to the RepB of plasmid pTXW from *L. paracasei* (ADD64219.1). Upstream of *repA* and *repB*, an IR of 9 bp with a 6 bp loop (5'-AGGGGGGGTACTACG/ACCCCCCCT-3') was noted. This IR and the underlined sequence embraced by the repeats coincide with the nick locus of the *dso* of RC-replicating plasmids of the pMV158 family (Ruiz-Masó et al., 2007). However, the presence of a set of proximal direct repeats (PDR) downstream of the nick site that could function as the binding locus of the *dso* (Ruiz-Masó et al., 2007) was not established. Moreover, thought several IRs were noted scattered through pRCID2.9, a region of dyad symmetry having a high potential of forming stem-loop structures that may function as the single strand origin of replication (*sso*) (del Solar et al., 1998) was not identified.

pRCEID3.2 consists of 3250 bp that encode 4 ORFs larger than 50 amino acids (Table 2). At the nucleotide level, pRCEID3.2 was shown to be almost identical (99% similarity) to pLA105 from *L. acidophilus* TK8912 (D49554.1; Kanatani et al. 1995b); the nucleotide similarity of both plasmids was complete for the first 1817 nt. Twenty eight nucleotide changes scattered in the second half of the molecules were observed, including single bp

312 insertions, deletions and nucleotide substitutions. Further, two single deletions in the sequence
313 of pRCEID3.2 were observed; their effect is to open the 3' end of the replicase gene (*repA*) a
314 little more than in pLA105. A small insert of 43 bp was noted in the sequence of pRCEID3.2
315 at position 2214, making the total length of the plasmid 30 bp longer than pLA105. The gene
316 product of ORF1 may consist of 162 amino acids having no homology to proteins held in
317 databases. The gene product of ORF2 may consist of 306 amino acids with a Rep_3
318 superfamily domain (pfam01051) that shows an amino acid similarity of over 70% to the
319 plasmid replication proteins of plasmids pREN (CBX32802), pLJ42 (AAZ13604) and
320 pSMB74 (NP_857600) from *Lactobacillus rennini*, *Lactobacillus plantarum* and *Pediococcus*
321 *acidilactici*, respectively. All these plasmids are thought to belong to the theta-replicating
322 plasmid family of pUCL287 (Benachour et al. 1997). Upstream of the *repA* gene of
323 pRCEID3.2, a putative *ori* region was observed, including a DR of 8 bp (CTCTTTTA)
324 repeated four times, and a tandem DR of 22 bp (ACAAATTGTCTGCTTATAGAAC)
325 repeated three times followed by a fourth truncated repeat (ACAAATTGTCTG). This
326 organization resembles that present in many theta-replicating plasmids of Gram-positive
327 bacteria (Kiewiet et al. 1993; Benachour et al. 1997). ORF3 encodes a peptide very similar to
328 the C-terminal part of a hypothetical protein of the *L. paracasei* plasmid pCD01
329 (AAW81266). Within this truncated protein, a perfect IR of 32 bp with a loop 12 nt long was
330 identified. This IR may be a remnant of an *sso* region similar to those present in RC-type
331 plasmids. This sequence might be related to ORF4, whose deduced product consists of 59
332 amino acids showing strong similarity to the N-terminal part of replication proteins of the
333 pC194-RC type plasmids, such as that of pNCD0151 (Z50861). In pLA105, this peptide has
334 been shown not to be involved in plasmid replication (Kanatani et al. 1995b).

Once again the nucleotide sequence of pRCEID13.9 was shown to be practically identical to that of pLA103 from *L. acidophilus* TK8912 (AB081463; Kanatani et al. 1995a), with only four nucleotide differences at positions 670, 8122, 8140 and 11784. Analysis of pRCEID13.9 sequence revealed the presence of 13 putative ORFs (Table 2). The most likely protein necessary for the replication of plasmid pRCEID13.9 was identified in ORF2. The gene product of ORF2 (a deduced 282 amino acid-long protein) showed a Rep_3 superfamily domain (pfam01051), completely identical to the so-called RepA1 protein of pLA103. Upstream of RepA1 of pRCEID13.9 two types of DR were seen, including an imperfect 8 bp DR (CCTCTTTA) repeated four times and a 22 bp DR (TTGGGTCTTTTTACGCGCTTAT) tandemly repeated 3.5 times. The nucleotide sequence of the 22 bp DR bears no similarity to that of the same length in plasmid pRCEID3.2. In pLA103, it has been proven necessary for plasmid replication (Kanatani et al. 1995c). ORF1 encodes a possible interrupted peptide similar to that of ORF4 from pRCEID3.2. ORF2 encodes a replicase, while ORF3 encodes a protein showing a DUF536 superfamily domain (pfam04394) of unknown function, but related to theta replication. The bacteriocin biosynthesis operon in pRCEID13.9 appears to cover four consecutive ORFs: ORF4, ORF5, ORF6 and ORF7 (Table 2). ORF7 encoded a short 46 amino acid peptide, which seems to be the structural gene of the bacteriocin. It is identical to acidocin A of plasmid pLA103 (NC_003458) (Kanatani et al. 1995a) and acidocin 8912 of plasmid pSJ2-8 (FM246455) (Kojic et al., 2010). ORF4 has been shown to code for the immunity protein (Kanatani et al. 1995a), and ORF5 and ORF6 seem to code for components of an ABC transporter that may be involved in the secretion of the bacteriocin.

The small nucleotide differences found among equivalent plasmids from TK8912 and TISTR1341 could be of help to track evolutionary changes of plasmids. As, for instance,

pRCEID3.2 showed many nucleotide changes with respect to pLA105, while molecules of pRCEID13.9 and pLA103, although larger, showed only minor differences.

3.5. Construction of shuttle vectors for *L. casei* and *E. coli*

To construct shuttle vectors for *L. casei* and *E. coli*, both the pRCEID2.9 (RC type) and pRCEID13.9 (theta type) replicons were used. Either replicon was cloned into pUC19E, a pUC19 derivative that does not replicate in *L. casei* but contains an erythromycin resistance gene allowing selection (Leenhoust et al. 1991). The pRCEID2.9-derived shuttle vector, designated pRCEID-LC2.9, consisted of a DNA sequence amplified with primers 1531-1558F and 187-213R containing the *ori*, *repA* and *repB* genes. The shuttle vector pRCEID-LC13.9 contained the *ori* and *repA1* sequences of pRCEID13.9, which was amplified by PCR with the primers p13.9-F1 and pRep13.9-R1. The cloning steps undertaken to obtain these vectors are shown in Figure 2. Cloning was accomplished in *E. coli*, after which constructs were transformed into *L. casei*. Electroporation of the constructs in *L. casei* ATCC393, BL23 and *L. casei* RCEID02 proved all four replicate in these strains. Transformation frequency of the vectors per μg of DNA ranged among the different hosts from $7.18x10^3$ to $1.3x10^4$ for pRCEID-LC2.9 and from $5.12x10^3$ to $1.0x10^4$ for pRCEID-LC13.9. As expected, pRCEID-LC2.9 and pRCEID-LC13.9Tc were shown to replicate stably in the same cell without antibiotic selection.

3.6. Segregational and structural stability of the shuttle vectors

The stability of the pRCEID-LC2.9 and pRCEID-LC13.9 constructs was assayed in *L. casei* RCEID02. In the absence of selective pressure, both vectors were maintained at percentages of 84 and 90, respectively, after 100 generations (Figure 3). The structural

stability of the constructs was studied by restriction analysis. Constructs were isolated every 20 generations under non-selective conditions and digested with several restriction enzymes. The plasmids had the same molecular size as the original vector and gave the same digestion patterns. These results suggest that pRCEID-LC2.9 and pRCEID-LC13.9 have good segregational and structural stability in *L. casei*. Segregational and structural analysis of the constructs was also checked in *E. coli*. While pRCEID-LC13.9 was rather stable after 100 generations, pRCEID-LC2.9 proved to be very unstable at both the segregational (32% maintenance) and structural (100% rearranged vectors) level.

3.7. Relative plasmid copy number of the constructs

The relative copy number per chromosome equivalent of pRCEID-LC2.9 and pRCEID-LC13.9 was measured by Q-PCR using exponentially growing cells. A ten-fold serial dilution of total DNA of the recombinant *L. casei* harboring either pRCEID-LC2.9 or pRCEID-LC13.9 was used to determine standard curves for the *repB*, *repA1* and *greA* genes. The curves obtained for *repB*, *repA1* and *greA* genes were linear (R^2>0.99) over the tested range. For pRCEID-LC2.9, the slopes of the curves for *repB* and *greA* genes were 3.23 and 3.24 respectively, and the average ΔC_T value was 3.93. For pRCEID-LC13.9, the slopes were 3.50 for both the *repA1* and *greA* genes, and a ΔC_T of 2.12. Assuming maximum and identical amplification efficiencies of target and reference genes, the relative copy numbers per chromosome equivalent of pRCEID-LC2.9 and pRCEID-LC13.9 were about 15 and 4 copies, respectively.

3.8. Expression of a green fluorescent protein (GFP) gene in *L. casei*

The usefulness of the pRCEID-LC13.9 vector was analyzed by expressing in both *E. coli* and *L. casei* a GFP encoding gene under the lactate dehydrogenase (LDH) promoter of *L. casei*. To this end, the LDH promoter was amplified with primers LDF_F and LDH_R using as a template total DNA from *L. casei* ATCC393. The amplicon was then cloned in pGEM-T Easy vector, resulting in the construct pLDH-PRO1. The green fluorescence protein gene (GFPuv), a variant of the wild gene from *Aequorea victoria*, was obtained from the commercial vector pGFPuv (Clontech) by double *Hin*dIII/*Spe*I digestion and cloned in pLDH-PRO1 digested with the same enzymes. The LDH-PRO1:GFPuv fusion segment was obtained by a double digestion of the construct with *Aat*II/*Spe*I and cloned in pRCEID-LC13.9 digested with the same enzymes. The resulting construct, pLC13.9:LDH-PRO1:GFPuv, was obtained in *E. coli* and electrotransformed into *L. casei*. Examination of the cells through the growth cycle under a fluorescence microscope showed expression of the GFP in both hosts. Strong fluorescence was recorded in more than 85% of the cells during exponential growth phase, while the percentage dropped to less than 20% after reaching the lag phase. Stability analysis in *L. casei* RCEID02 proved the construct to be maintained after 100 generations at a similar percentage (88%) to that of pRCEID-LC13.9.

Introduction of this construct in *L. casei* could allow gene expression analysis, but also tracking of cells by fluorescence during manufacturing and ripening of fermented products and/or during probiotic trials.

4. Conclusions

In conclusion, three plasmids from the *L. casei* TISTR1341 were sequenced and analyzed in this work. The fact that all three plasmids proved to be almost identical to plasmids from the *L. acidophilus* TK8912 strain suggests that these two strains are very strongly related, if

not the same. Two of the replicons of the *L. casei* TISTR1341 were used to construct a first generation of cloning vectors, which may be used to complement those currently in use. These new vectors were shown to replicate in different *L. casei* strains, including the model strains ATCC393 and BL23, in which they proved to be stable at both the structural and segregational level. Additionally, since the vectors based on pRCEID 2.9 and pRCEID13.9 showed different copy numbers, this might help fine-tune the copy number of cloned genes in this species. Finally, pRCEID-LC13.9 was used for the cloning and expression of a gene encoding a green fluorescent protein in *L. casei* RCEID02 under the promoter of the lactate dehydrogenase gene. Basic replicons from co-resident plasmids from a single cell allow the development of more robust naturally compatible plasmid vectors.

The pyrosequencing technique seems to be a convenient approach for plasmid analysis in LAB, among which strains of many species such as *L. casei* are found to contain multiple plasmid molecules.

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Table1.- Bacterial strains, plasmids and oligonucleotide primers utilized in this study.

Materials	Relevant properties	Source or reference
Bacteria		
Escherichia coli XL1-Blue	White/blue screening	Stratagene, La Jolla, CA
E. coli GM2929F'	Transformation host	Stratagene, La Jolla, CA
Lactobacillus casei TISTR1341	Native plasmid-containing strain (isolated from chicken faeces)	TISTR[a]
L. casei RCEID01	*L. casei* TISTR1341 derivative, pRCEID13.9-cured strain	This study
L .casei RCEID02	*L. casei* TISTR1341 derivative, plasmid-free strain	This study
L. casei ATCC393	Plasmid free strain	ATCC[b]
L. casei BL23	Plasmid free strain	Acedo-Félix and Pérez-Martínez, 2003
Lactobacillus sakei CECT906	Bacteriocin sensitive strain	CECT[c]
Lactobacillus rhamnosus GG	Human isolate (ATCC53103)	ATCC
Bifidobacterium longum H66	Source of *tet*(W) resistance gene	Laboratory Collection
Plasmids		
pUC19E	Ap^r, Em^r, pUC19 carrying the Em^r gene of pE194 at the *Sma*I site	Leenhouts et al. (1991)
pGEM-T Easy	Ap^r, M13ori, T-overhang cloning vector	Promega, MD, USA
pGFPuv	Commercial vector having a GFP gene variant of *Aequorea victoria* for maximal UV fluorescence	Clontech, CA, USA
pRCEID-LC2.9	Ap^r, Em^r, *E. coli-L. casei* shuttle vector based on pRCEID2.9	This study
pRCEID-LC13.9	Ap^r, Em^r, *E. coli-L. casei* shuttle vector based on pRCEID13.9	This study
pRCEID-LC13.9EmTc	Ap^r, Em^r, Tc^r, pRCEID-LC13.9 derivative, the Tc^r gene was inserted at *Sac*I site	This study
pRCEID-LC13.9Tc	Ap^r, Tc^r, pRCEID-LC13.9EmTc derivative, the Em^r gene was removed by *Sal*I digestion	This study
pLC13.9:LDH-PRO1:GFPuv	pRCEID-LC13.9-derived construct expressing the GFPuv gene under the lactate dehydrogenase promoter of *L. casei*	This study
Oligonucleotides	Sequence (5'-3')	
27F	agagtttgatcctggctcag	S-D-Bact-0008-a-S-20
1492R	ggttaccttgttacgactt	S-*-Univ-1492R-b-A-21
M13_F (-21)	tgtaaaacgacggccagt	Laboratory primer
M13_R (-27)	ggaaacagctatgaccatg	Laboratory primer
Gap_p2.9_F	cgtacgttacaacgtcactca	This study
Gap_p2.9_R	cgggatatgatccgctccaa	This study
p3.2gap-F1	gggtttggggagagattctc	This study
p3.2gap-R1	gtgcggtttggtttgtcgtg	This study
p3.2gap-F2	gcattaactcagcgctttc	This study
p3.2gap-R2	gatcgaccacttttgaggc	This study
1531-1558F	gatatggatccagaaagaaaacaaaagc (*Bam*HI)	This study
187-213R	cacaaagtctggatcctgcgaggcatg (*Bam*HI)	This study
p13.9-F1	caccgaagcttcagctgaggttc (*Hin*dIII)	This study
pRep13.9-R	gtaaaagcttaaacagctggagacaccc (*Hin*dIII)	This study
TEF-F1	cggaatatagcgctgctaag	This study
TEF-R1	gccaatatcggcaatatcac	This study
RepB2.9-F1	actggcatatagcgatgttt	This study
RepB2.9-R1	agtaccgaatcattccctg	This study
RepA13.9-F1	agcaattggacaagattcag	This study
RepA13.9-R1	tcgaaattcctgcatgctaa	This study
tetWsacF	ccctggagctcatgctcatgtac (*Sac*I)	Flórez et al. (2006)
tetWsacR	ccatcggagctccataacttctg (*Sac*I)	Flórez et al. (2006)
LDH_F	atgggaattcagcttttagtcctcgtga (*Eco*RI)	This study
LDH_R	gcccaagctttatccgtaatacttgcc (*Hind*III)	This study

[a]TISTR, Thailand Institute of Scientific and Technological Research.
[b]ATCC, American Type Culture Collection.
[c] CECT, Colección Española de Cultivos Tipo (Spanish Type Culture Collection).
Underlined nucleotides show introduced restriction enzyme sites, which are indicated in parenthesis.

Table 2.- Open reading frames (ORFs) identified in plasmids pRCEID2.9, pRCEID3.2 and pRCEID13.9 from *L. casei* TISTR1341.

Plasmid *gene/ORF*	5' end position	3' end position	%GC content	No. of aa	Known protein with the highest homology (microorganism)	% aa identity (length)	GenBank Accession no.
pRCEID2.9							
mob	307	1617	45	436	Mob of pTXW (*L. casei*) Mob of pLA106 (*L. acidophilus*)	99 (432) 90 (394)	ADD64221.1 BAA21093.1
repA	1983	2138	35	51	RepA of pLA106 (*L. acidophilus*)	100 (51)	BAA21094.1
repB	2195	2775	42	193	RepB of pLA106 (*L. acidophilus*)	100 (193)	BAA21095.1
pRCEID3.2							
ORF1	244	732	53	162	Homology to other proteins was not found	-	-
repA	1058	1978	43	306	RepA of pREN (*L. rennini*) RepA of pLKS (*L. plantarum*) RepA of pUCL287 (*Pediococcus acidilactici*)	72 (218) 75 (214) 69 (200)	CBX32802 BAA87064.1 CAA53278
ORF3	2712	2963	41	83	Hypothetical protein of pCD01 (*L. paracasei*)	84 (70)	YP_003329270.1
repA2	3108	37	46	59	Replication protein A2 of pLA103 (*L. acidophilus*) Replication protein of pNCDO151 (*L. casei*)	76 (45) 74 (44)	BAB86315.1 CAA90731.1
pRCEID13.9							
repA2	2114	2290	44	59	Replication protein A2 of pLA103 (*L. acidophilus*) Replication protein of pLR001 (*L. rhamnosus* HN001) Putative replication protein A of pCD01 (*L. paracasei*)	100 (59) 96 (57) 96 (57)	BAB86315 ACH91615.1 AAW81267.1
repA1	2935	3783	38	282	Replication protein A1 of pLA103 (*L. acidophilus*)	100 (282)	BAB86316
repB[a]	4478	3972	44	169	Replication protein B of pLA103 (*L. acidophilus*)	100 (169)	BAB86317
ORF4	5619	5927	36	103	Immunity protein of pSJ2-8 (*L. paracasei*) Immunity protein of pLA103 (*L. acidophilus*)	100 (103) 100 (103)	ZP_04673880 BAB86319
ORF5	6320	8476	40	719	ABC transporter protein of pSJ2-8 (*L. paracasei*) ABC transporter protein of pLA103 (*L. acidophilus*)	99 (694) 99 (690)	YP_002720031 BAB86320
ORF6	8490	9863	41	458	Accessory protein of pLA103 (*L. acidophilus*) Accessory protein of pSJ2-8 (*L. paracasei*)	100 (458) 99 (359)	BAB86321 YP_002720030
ORF7	10025	10162	38	46	Acidocin 8912 of pSJ2-8 (*L. paracasei*) Acidocin A of pLA103 (*L. acidophilus*)	100 (46) 100 (46)	BAB86322 YP_002720029

ORF8	10497	10871	31	125	Hypothetical protein of pLA103 (*L. acidophilus*) Hypothetical protein of pSJ2-8 (*L. paracasei*)	100 (125) 100 (125)	BAB86323 YP_002720028
ORF9[a]	11429	11031	44	128	Transposes pLA103 (*L. acidophilus*)	100 (132)	BAB86324
ORF10[a]	11726	11508	40	72	Transposes pLA103 (*L. acidophilus*)	100 (72)	BAB86325
ORF11	12414	11911	43	167	Hypothetical protein of pLA103 (*L. acidophilus*)	98 (113)	BAB86326
ORF12	12572	13468	37	298	Hypothetical protein of pLA103 (*L. acidophilus*)	85 (254)	BAB86327
ORF13[a]	13565	13906	44	113	Hypothetical protein of pLA103 (*L. acidophilus*)	100 (113)	BAB86328

2 [a]These ORFs are encoded on the complementary strand.

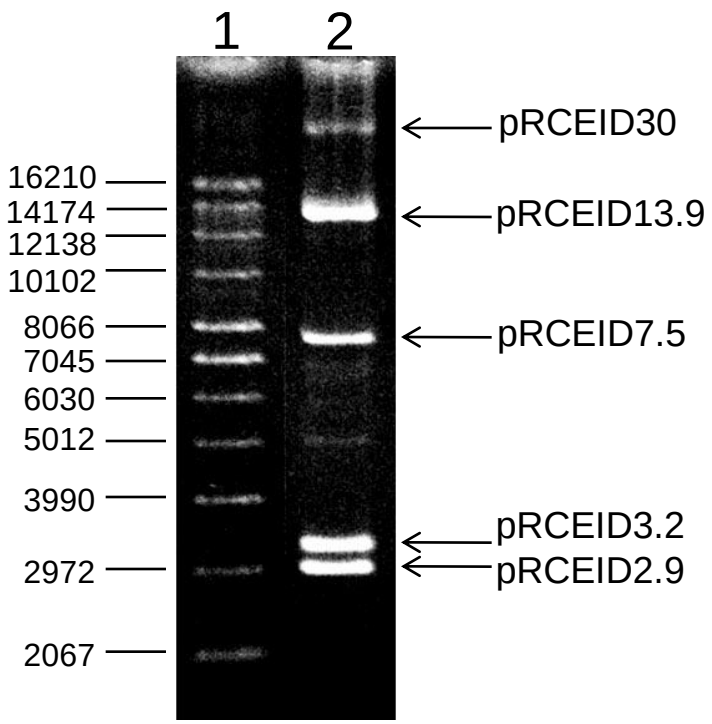
1
2
16210
14174
12138
10102
8066
7045
6030
5012
3990
2972
2067
pRCEID30
pRCEID13.9
pRCEID7.5
pRCEID3.2
pRCEID2.9

Figure 1

Figure 2

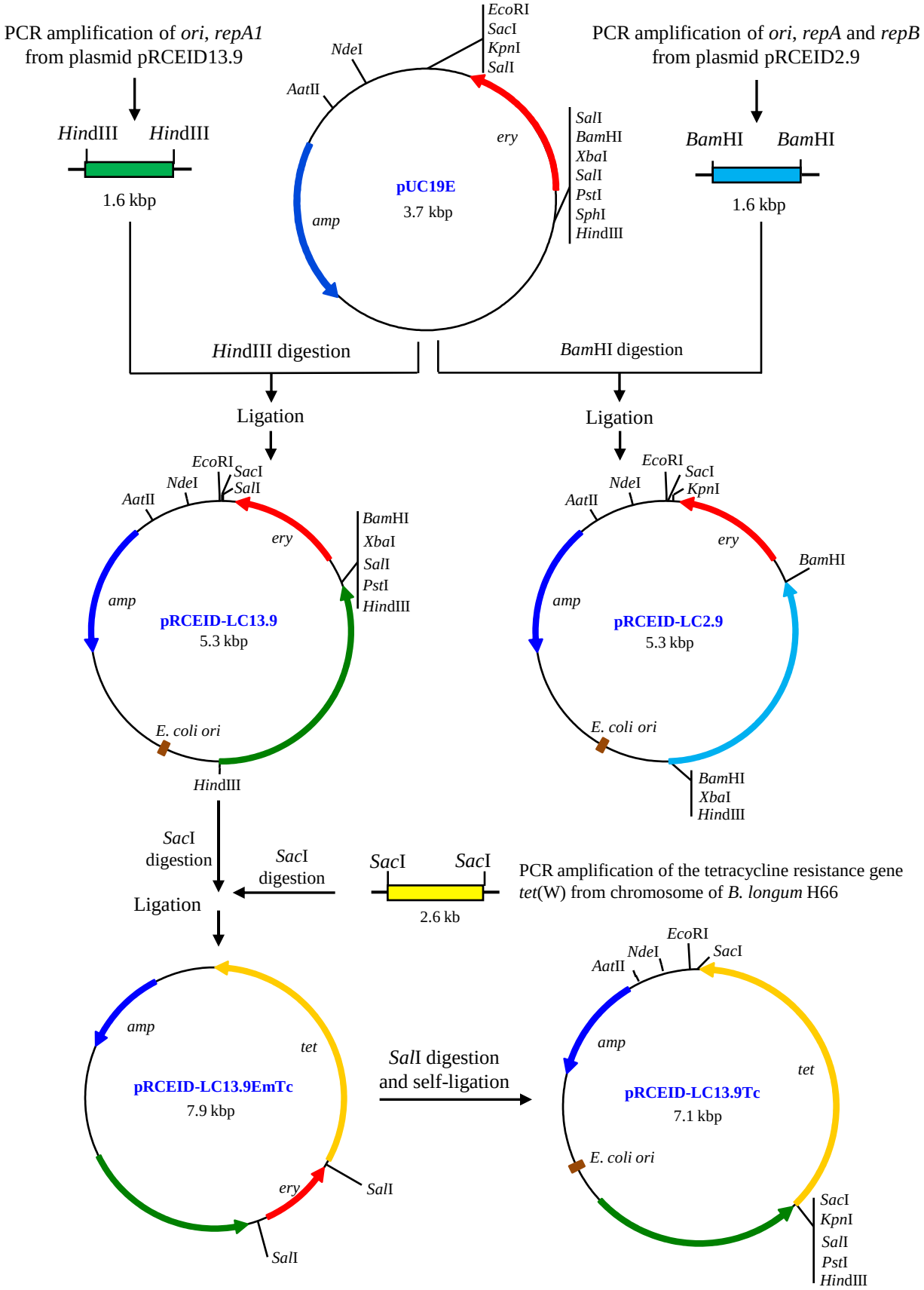

Figure 3

Figure 3

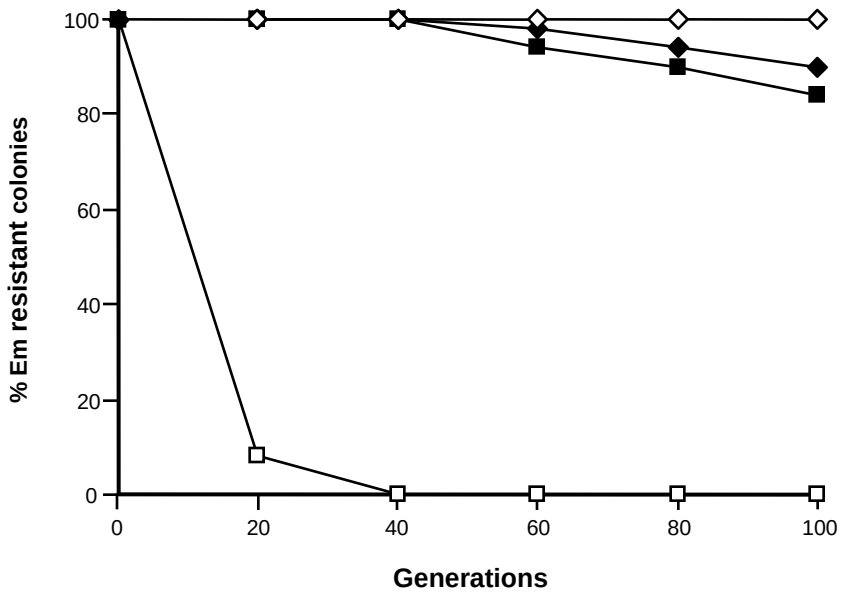

Figure 4

FIGURE CAPTIONS

Figure 1.- Agarose gel electrophoresis of the plasmid profile of *L. casei* TISTR1341 strain. Lane 1 is a super coiled DNA ladder (Invitrogen, Carlsbad, CA). On the left, size in bp of the different molecules of the ladder. Lane 2, total plasmid DNA isolated from *L. casei* TISTR1341, indicating the name and position of the different plasmids by arrows.

Figure 2.- Construction of the shuttle vectors, pRCEID-LC2.9, pRCEID-LC13.9, pRCEID-LC13.9EmTc and pRCEID-LC13.9Tc. *amp*, *ery*, and *tet*, indicates ampicillin, erythromycin and tetracycline resistance genes, respectively.

Figure 3.- Segregational stability of pRCEID-LC2.9 (squares) and pRCEID-LC13.9 (rhombus) vectors in *E. coli* and (open) *L. casei* (filled). Results are average of three independent experiments. Em, erythromycin.