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RESEARCH NOTE

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Screening of a Novel Lactobacilli Replicon from Plasmids of *Lactobacillus reuteri* KCTC 3678

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Abstract A novel lactobacilli replicon from plasmids of *Lactobacillus reuteri* KCTC 3678 was isolated. Eight *L. reuteri* strains from Korean Collection for Type Cultures (KCTC) and Korea Food Research Institute (KFRI) were screened for cryptic plasmids and most strains harbored 1 or 2 plasmids. Particularly, *L. reuteri* KCTC 3678 contained 6 plasmids which all were used for screening of lactobacilli replicon. *Eco*RI digests of the plasmid DNA prep from *L. reuteri* KCTC 3678 were ligated with pUC19 and the recombinant DNAs were serially named from pLR1 to pLR7. A *cat* (chloramphenicol acetyltransferase; Cm^r) gene originated from pC194 was introduced into pLR1-7, resulting in pLR1*cat*-pLR7*cat*, respectively. The recombinant plasmids were introduced into *L. reuteri* KCTC 3679, and only transformants harboring pLR5*cat* were obtained, indicating that the insert in pLR5 functioned as a lactobacilli replicon.

Key words: *Lactobacillus reuteri*, lactobacilli replicon, plasmid vector

Introduction

Strains of *Lactobacillus reuteri* have been found in gastro-intestinal (GI) tracts of humans and animals (1). Due to its beneficial and probiotic effects (2,3), researchers have paid attention to studies on its applications. Particularly, it has an ability to convert glycerol into 3-hydroxypropionaldehyde, known as reuterin, with a broad range antimicrobial activity against pathogens and food spoilage organisms (4), which might render it commercially important. Development of cloning vectors for *L. reuteri* is essential if basic and applied studies on *L. reuteri* are to be accelerated. To the best of our knowledge, few groups have studied on the development of cloning vectors for this species (1,5). In this study, we screened *L. reuteri* strains for cryptic plasmids and a lactobacilli replicon, which could be used for cloning vectors, from plasmids of *L. reuteri* KCTC 3678 was cloned and sequenced.

Materials and Methods

Bacterial strains, plasmids, and primers Bacterial strains, plasmids, and primers used in this study are shown in Table 1. *L. reuteri* strains were purchased from Korean Collection for Type Cultures (KCTC) and Korea Food Research Institute (KFRI). *Escherichia coli* strains were cultured in Luria-Bertani (LB) broth at 37°C with vigorous aeration and *L. reuteri* strains were grown in MRS broth at 37°C. For antibiotic selection, 100 μg/mL of ampicillin was used for *E. coli* and 20 and 5 μg/mL of chloramphenicol were used for *E. coli* and *L. reuteri*, respectively.

DNA manipulation General DNA manipulation techniques were used according to the methods in molecular cloning: a laboratory manual (8). To transform *E. coli*, a kit (Yeastern Biotech Co., Taipei, Taiwan) for heat shock method was used. In the case of *L. reuteri*, it was cultured in 200 mL of MRS broth until mid-exponential phase (OD_{600} 0.5-0.6), harvested by centrifugation with 2,500×g at 4°C for 10 min, and washed 3 times with ice-cold 10% glycerol. The washed cells resuspended with the same solution, 1/250 of original culture volume, were used for electrotransformation with a condition of 2.0 kV, 25 μF, and 200 Ω. For amplification of a *cat* gene from pPSAB1 (Table 1), a primer set, CAT_F and CAT_R, was designed by a web-based program and the polymerase chain reaction (PCR) was performed with T3 thermocycler (Biometra, Goettingen, Germany), where the cycle program of denaturation, annealing, and extension steps comprised of 1 cycle of 2 min at 95°C, then 30 cycles of 30 sec at 95°C, 30 sec at 57°C, and 1 min at 72°C, followed by 1 cycle of 5 min at 72°C.

DNA sequencing and the sequence analysis The nucleotide sequence of a putative lactobacilli replicon in pLR5 was analyzed. Basic sequence analyses were done by DNASTAR software programs (DNASTAR Co., Madison, WI, USA). Homology searches were performed by BLAST server at the National Centre for Biotechnology Information (NCBI). The sequence data were deposited in the GenBank database of NCBI (GenBank accession no. DQ786756).

Results and Discussion

Eight *L. reuteri* strains were screened for cryptic plasmids. Most strains harbored 1 or 2 big-sized plasmids. However, *L. reuteri* KCTC 3678 strain harbored 6 plasmids including middle-sized ones (lane 1 in Fig. 1). To obtain appropriate-sized DNA fragment including a lactobacilli replicon from the plasmids, we treated the plasmid DNA prep with

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Table 1. Bacterial strains, plasmids, and primers

	Characteristics	Source/reference
Strains		
E. coli JM109	F' *traD36 lacI*^{q} *(lacZ)M15 proA*$^+$*B*$^+$*/e14*$^-$ *(mcrA) (lac proAB) thi gyrA96 (Nal*^{r}*) endA1 hsdR17 (*r_k^- m_k^+*) relA1 supE44 recA1*	Yeastern Biotech Co.
L. reuteri KCTC 3678	Strain harboring plasmids used in this study	KCTC[1)]
L. reuteri KCTC 3679	Recipient host for pLR5*cat*	KCTC
Plasmids		
pUC19	Ap^r, ColE1 *ori*, 2.68 kb	6
pPSAB1	Ap^r, Cm^r, template DNA for the amplification of a *cat* gene, 8.6 kb	7
pLR1-7	Recombinant DNAs where *Eco*RI digests of the plasmid prep from *L. reuteri* KCTC 3678 were randomly inserted into pUC19	This study
pLR1-7*cat*	A *cat* gene was introduced into pLR1-7, respectively	This study
Primers		
CAT_F	5'-TGCACTGCAGTGCACGGCAATAG-3'[2)]	This study
CAT_R	5'-TGGGCCTGCCTCTCCTGCAG-3'	This study

[1)]Korean Collection for Type Cultures.
[2)]The nucleotides underlined in the primer sequences are *Pst*I enzyme sites.

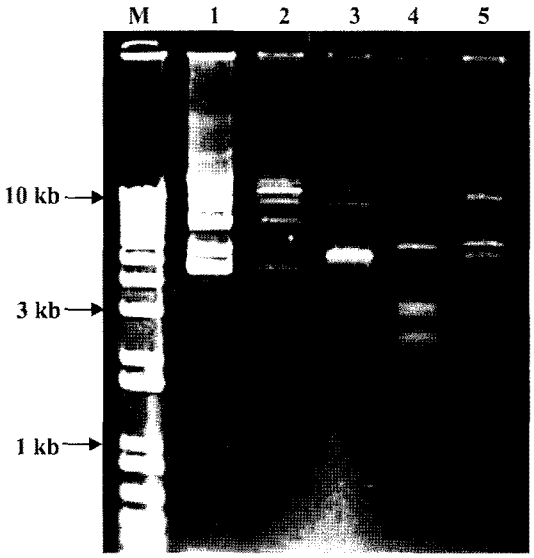

Fig. 1. Restriction profiles of plasmids from *Lactobacillus reuteri* KCTC 3678. M, 1 kb DNA ladder (Invitrogen Co.); lane 1, CCC (covalently closed circular) typed plasmids; plasmids treated with *Bam*HI (lane 2), *Eco*RI (lane 3), *Hind*III (lane 4), and *Pst*I (lane 5).

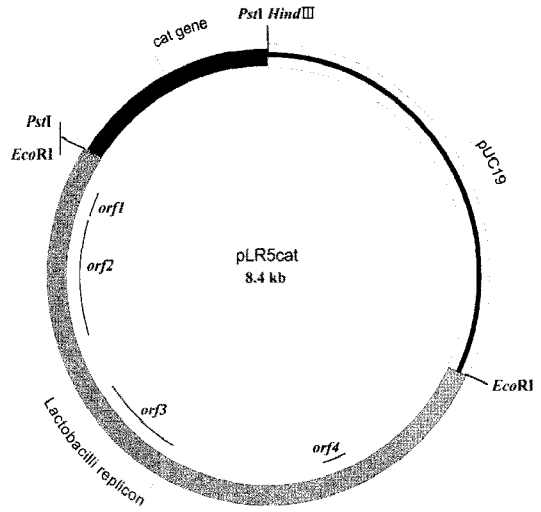

Fig. 2. Physical map of pLR5*cat* and putative *orfs* of the lactobacilli replicon.

*Eco*RI (Fig. 1) or *Hind*III enzyme producing small and middle-sized DNA fragments. DNA fragments digested by *Eco*RI were randomly inserted into pUC19 and the recombinant DNAs were serially named from pLR1 to pLR7. And a *cat* gene amplified by PCR using pPSAB1 as template with primer set, CAT_F and CAT_R, was introduced into pLR vectors, respectively for antibiotic selection of *L. reuteri* transformants and named pLR1-7*cat*. The 7 recombinant DNAs, pLR1-7*cat*, were separately transferred to electrocompetent *L. reuteri* KCTC 3679. Only transformants harboring pLR5*cat* were selected on MRS agar plate supplemented with 5 μg/mL of chloramphenicol with transformation efficiency of 3×10^3 cells/μg DNA, indicating the insert DNA of pLR5 functioned as a lactobacilli replicon. The recombinant DNA from *L. reuteri* KCTC 3679 was compared with pLR5*cat* from *E. coli* and we confirmed the restriction profiles corresponded to each other on an electrophoresis gel (data not shown). The physical map of pLR5*cat* is shown in Fig. 2 and the nucleotide sequence of the insert of pLR5 was determined. *orf1* and *orf2* showed no significant similarity to the genes from databases at NCBI. Orf3, a putative recombinase, consisting of 202 amino acids was homologous to a superfamily of recombinase/resolvase (9,10) and showed the highest similarity (42% identity) to a recombinase from conjugative plasmid pAM373 of *Enterococcus faecalis* (GenBank accession no. NP_072000) (11). Orf3 was also predicted to have a helix-turn-helix DNA-binding domain, indicating it could be an enzyme to contribute the segregation stability of plasmid by reducing the number of plasmid multimers from homologous recombination (12). Orf4, a putative replication protein, consisting of 57 amino acids was homologous to Rep proteins from *Lactobacillus* spp. and showed the best match with a putative replication protein (ORF9) from pCD01 of *L. paracasei* NFBC338 (36% identity; GenBank accession no. AAW81271) followed

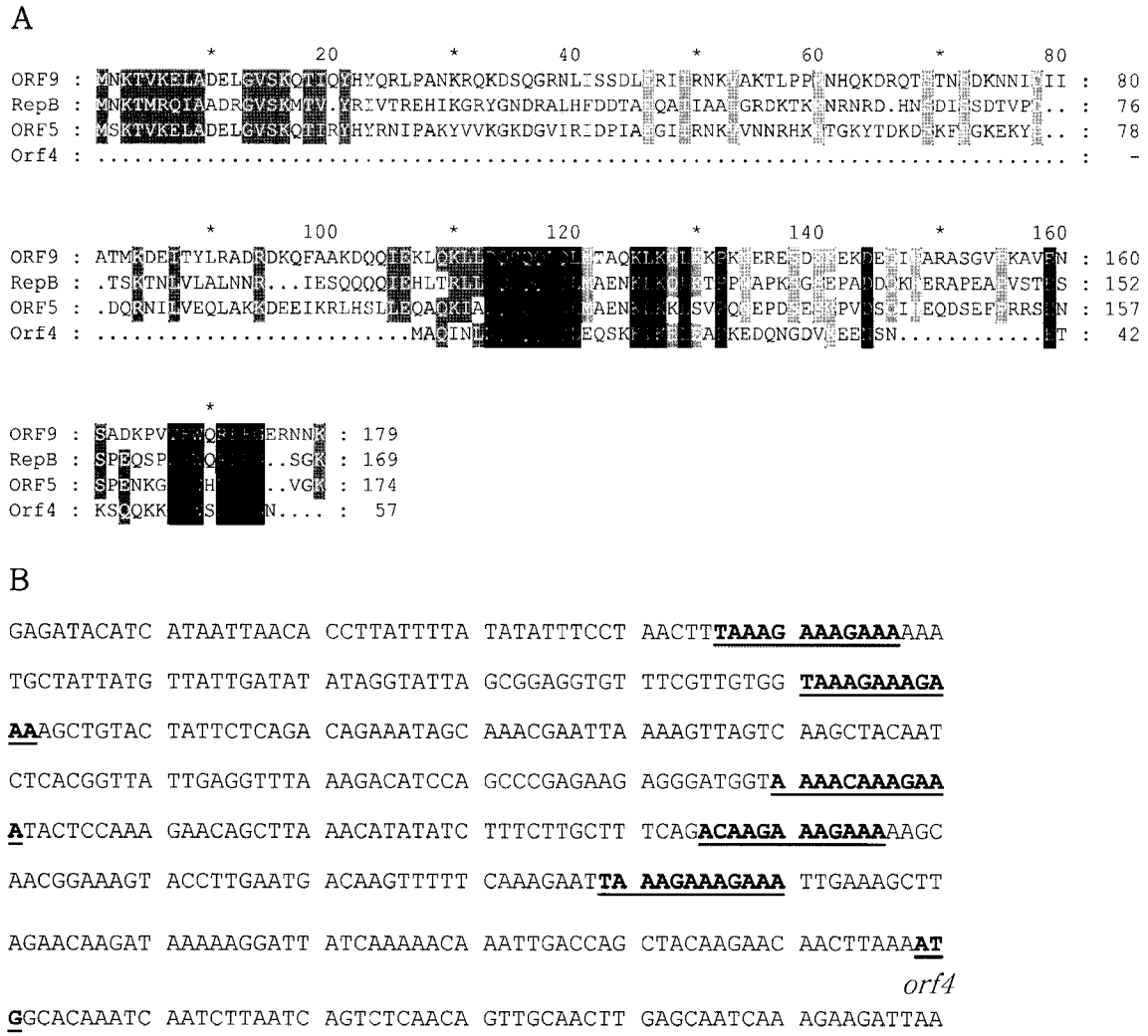

Fig. 3. (A) Alignment of the putative Rep protein (Orf4) of the lactobacilli replicon with those from other lactobacilli plasmids. ORF9 from pCD01 (GenBank accession no. AY662330), RepB from pLA103 (NC_003458), and ORF5 from pCD02 (AY662331). The protein sequences were aligned at GeneDoc program (http://www.nrbsc.org/). Shading indicates the extent of sequence conservation (black 100%, dark grey 80%, and light grey 60%). (B) The putative *ori* region of the lactobacilli replicon. The 5 direct repeats (12 bp) and the start codon (ATG) of *orf4* are underlined.

by RepB from pLA103 of *L. acidophilus* TK8912 (39% identity; NP_604409) and replication protein B (ORF5) from pCD02 of *L. paracasei* NFBC338 (34% identity; AAW81290) (13,14). The multiple sequences alignments of the putative Rep proteins are shown in Fig. 3A. Although Orf4 is homologous to RepB from pLA103 which replicates via theta mechanism, we could not postulate the replication mechanism of the lactobacilli replicon because the sequence does not contain a gene for RepA typical in theta mechanism discussed in previous paper (13). We also could not detect the sequence of double-strand origin (DSO) for rolling circle replication located on upstream of *rep* genes (15), however, a putative *ori* region consisting of 5 direct repeats (12 bp) existed on upstream of *orf4* (Fig. 3B).

The works performed in this study are prerequisite to develop a successful cloning vector for lactobacilli, mainly *L. reuteri*. In the future, the lactobacilli replicon in pLR5 will be characterized and genetic components such as strong promoter, terminator, and multiple cloning sites (MCS) will be introduced into pLR5*cat*. Additionally, a development of food-grade vector using the lactobacilli replicon would be considered (16). Again, *L. reuteri* must be one of the most important bacteria for commercial aspects (17) and the development of cloning and expression vectors for this species is essential to amplify the bacterial potentialities.

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