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ARTICLE

Complete sequence and gene analysis of a cryptic plasmid pLU4 in *Lactobacillus reuteri* strain LU4 (KCTC 12397BP)

Da-He Kim[1] · Young-Ju Jeon[1] · Myung-Jun Chung[2] · Jae-Gu Seo[2] · Young-Tae Ro[1]

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Abstract A cryptic plasmid, pLU4, was isolated and sequenced from *Lactobacillus reuteri* strain LU4, a probiotic strain isolated from human feces in Korea. pLU4 was 33,411 bp in size with a G + C content of 40.1% and contained 41 putative open reading frames (ORFs) of more than 120 bp. Functions were assigned to 24 of these ORFS by a homologous sequence search and domain characterization, and the rest were annotated as conserved hypothetical proteins or hypothetical proteins, based on highly similar proteins found in other *Lactobacillus* species. A region encoding a cluster of conjugative transfer genes (*trs* or *tra*) in pLU4 showed high similarity and co-linearity with the *trs* region of plca36 and *tra* region of pLgLA39 from *L. casei* str. Zhang and *L. gasseri*, respectively. This is the first report of a *trs* cluster sequence from plasmids found in *L. reuteri* species. Comparative gene analysis revealed that pLU4 also encodes highly conserved genes for plasmid replication and stability found in several other *Lactobacillus* plasmids, indicating that plasmids from the genus *Lactobacillus* may contribute to horizontal gene transfer and adaptation to the environment. The findings of this study provide important information about these industrially relevant phenotypes and give insight into the structure, function, and evolution of large *Lactobacillus* conjugative plasmids.

✉ Young-Tae Ro
ytaero@kku.ac.kr

[1] Laboratory of Biochemistry, Graduate School of Medicine, Konkuk University, Chungju 27478, Korea

[2] R&D Center of Cell Biotech Co., Ltd., Gimpo 10003, Korea

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Introduction

Lactobacillus reuteri is a hetero-fermentative lactic acid bacterium that naturally resides in the gastrointestinal tracts of humans and other animals [1] and is considered as indigenous *Lactobacillus* species in humans with *L. gasseri* [2]. *L. reuteri* is known to have probiotic properties [3, 4] and to produce a potent antibacterial substance, reuterin, that inhibits a broad spectrum of microorganisms, including bacteria, fungi, and protozoa [5]. In addition, *L. reuteri* strains have been found to modulate the severity of enteric infections and intestinal immune responses [6, 7].

Generally, most *Lactobacillus* strains harbor one or more plasmid species, the size of which can vary from 1.2 to 150 kb [8]. Although many of the plasmids are cryptic, some important functions that relate to lactose metabolism, antibiotic resistance, bacteriocin production, and immunity, DNA restriction and modification, exopolysaccharide production, *N*-acetyl glucosamine fermentation, and transport of certain amino acids (e.g., cysteine) have been found to be encoded by plasmids [9, 10]. Three strains of *L. reuteri* were characterized as having plasmids, among which 18 plasmids of various sizes (1.87–53.0 kb) have been sequenced at the time of this writing (https://www.ncbi.nlm.nih.gov/genome/plasmids/438). Some of these plasmids carry genes that may contribute to environmental adaptation of the host cell, as these genes were found to encode proteins associated with plasmid replication,

antibiotic resistance, amino acid or sugar transport, and restriction-modification system [11–14].

L. reuteri strain LU4 (KCTC 12397BP) was isolated from human feces in Korea and found to exhibit probiotic traits such as anti-inflammatory activity. The expression of pro-inflammatory cytokines, e.g., interleukin-1β (IL-1β) and IL-6, was suppressed in rat splenocytes, indicating that strain LU4 could be probiotic. In addition, strain LU4 was found in a preliminary experiment to have at least one plasmid species, estimated to be approximately 30 kb based on restriction mapping. In relation to this, we questioned whether the plasmid includes some genes associated with strategies for survival in the environment and probiotic properties. In the present study, we report the entire nucleotide sequence and results of a detailed genetic analysis of the large plasmid, named pLU4, in *L. reuteri* strain LU4, which encodes genes primarily associated with conjugation, plasmid replication, and stability. This is the first sequencing and gene analysis reports of a plasmid from *L. reuteri* strains that possesses a cluster of conjugative transfer genes, indicating a potential role for the pLU4 plasmid in horizontal gene transfer and adaptation to the environment.

Materials and methods

Bacterial growth and plasmid DNA isolation

L. reuteri strain LU4 was grown in MRS medium (Becton–Dickinson, USA). Plasmid DNAs were prepared from *L. reuteri* cells by the standard alkaline sodium dodecyl sulfate method after limited cell-wall hydrolysis [15].

Sequence determination and analysis of pLU4

The isolated pLU4 was digested with suitable restriction enzymes and cloned into multiple cloning sites of a pBluescript II SK^+ phagemid cloning vector to create a plasmid library. The resulting library clones were then used as templates for sequencing by using by the dideoxy-chain termination method. DNA sequencing was performed with the 96-capillary Applied Biosystems® 3730xl DNA Analyzer at Cosmo Genetech (Seoul, Korea), initially using standard M13-20 and M13 reverse primers. Multiple sequence alignments were performed using the ClustalW2 web service (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and manually edited. For a complete sequence determination of pLU4, primer walking was performed subsequently with internal primers designed by using the sequence information obtained from previous sequencing runs.

Potential coding regions were determined by Glimmer3 [16], and open reading frames (ORFs) of >120 bp were retained. The nucleotide sequence and deduced ORFs were analyzed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved domains of the putative ORFs were identified in the Conserved Domain Database (CDD) of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The nucleotide sequence of pLU4 has been deposited in GenBank database under accession no. KM063576.1.

For comparative analysis, five plasmids were downloaded from the NCBI Web site. Their accession numbers are CP000935.1 (*L. casei* str. Zhang plca36), AB436615.1 (*L. gasseri* LA39 pLgLA39), CR377166.1 (*L. plantarum* WCFS1 pWCFS103), AVAB01000112.1 (*L. fermentum* MTCC 8711 pLF03), and AE001272.1 (*Lactococcus lactis* strain DPC3147 pMRC01).

Results and discussion

DNA sequence and gene organization of pLU4

pLU4 is a circular plasmid of 33,411 bp with an average G + C content of 40.1%, which is slightly higher than that reported previously for *L. reuteri* chromosomal DNA (~39% G + C) [1, 17]. Forty-one ORFs greater than 120 bp in length were identified and their average lengths were 716 bp, accounting for 88% of the plasmid sequence (Fig. 1; Table 1). All genes, except seven putative genes, were transcribed in the same direction. Functions of 24 potential genes were assigned by a homologous sequence search and domain characterization, whereas another thirteen and four genes were simply designated as conserved hypothetical protein (CHP) or hypothetical protein (HP), respectively (more detailed descriptions are available in Table 1). Analysis of the gene organization of pLU4 suggests that the plasmid can be divided into two functional regions: a gene cluster for conjugative transfer and another for plasmid replication and maintenance.

Gene cluster for conjugative transfer

The pLU4 plasmid contains a region of more than 19 kb encoding homologs of conjugal transfer genes almost exclusively from lactic acid bacteria. Approximately 24 putative ORFs between *orf3* (nt position 1,710–1,895) and *orf26* (*ltrC*, nt position 19,803–20,927) occur within the region, of which 14 products were assigned putative conjugal transfer functions (Table 1). Significantly, all of the ORFs within this region, except for putative *orf5* and *orf6*, are transcribed in the same direction, suggesting an operon-style scheme that is consistently found in conjugal transfer systems [18, 19]. The gene organization of this region in

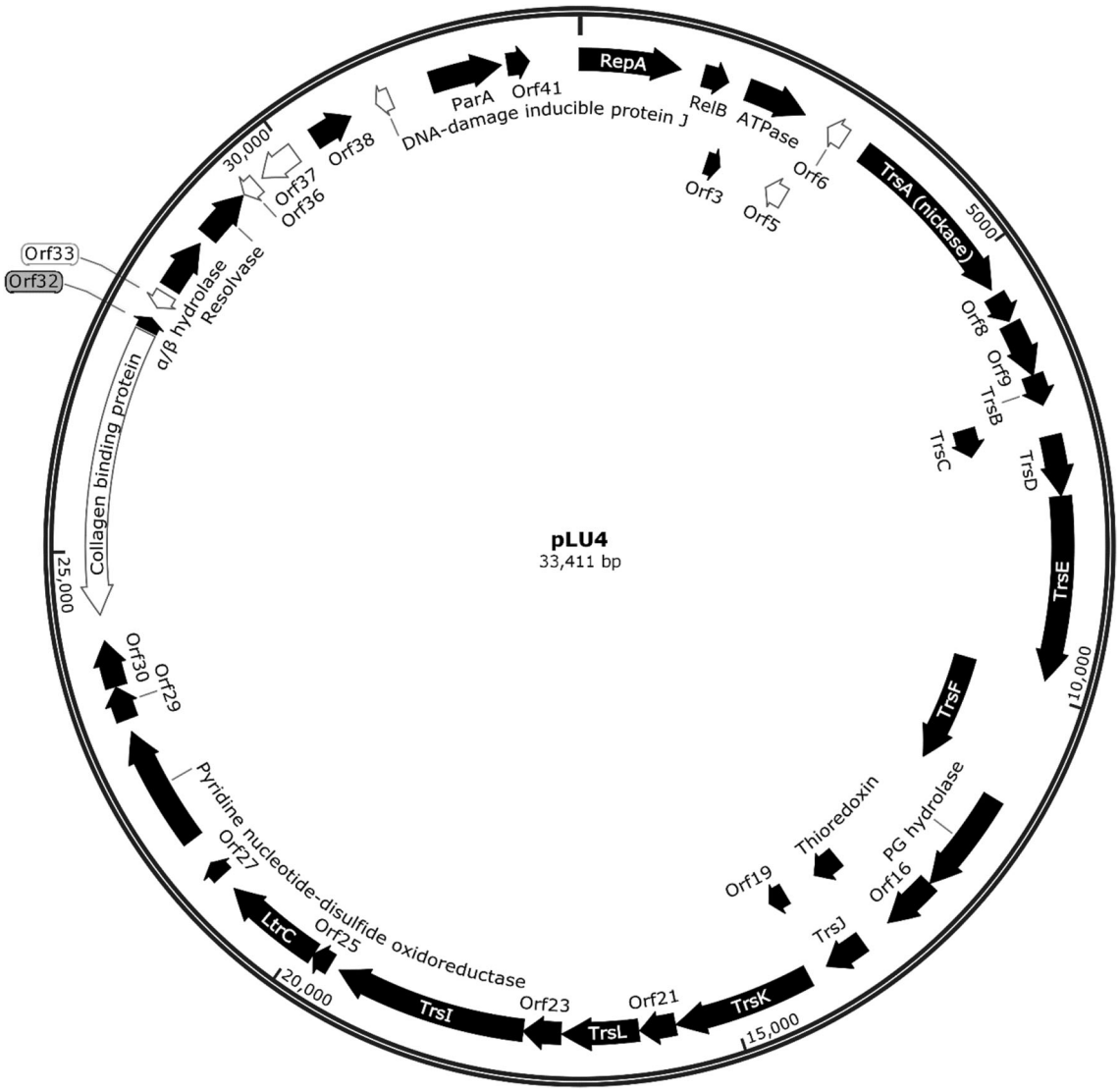

Fig. 1 General features of plasmid pLU4. The *outer circle* shows the bp scale. The *arrows* in the *inner circles* show the 41 putative genes or open reading frames (ORFs). The *colors* (*black and white*) of *arrows* indicate their translation orientations

pLU4 is very similar to the corresponding regions of several lactic acid bacterial plasmids (Fig. 2). The 10 *trs* gene products (namely TrsA-F, TrsJ-L, and TrsI) of pLU4 are highly similar to those (*trs* or *tra*) of *L. casei* str. Zhang plca36 (96–100% identical [20], *L. gasseri* pLgLA39 (88% to 100% identical [21], and *L. plantarum* pWCFS103, (67–100% identical [22] (Table 2). These gene products, except for TraA, also have homologs in *L. fermentum* MTCC 8711 pLF03 (43–86% identical [23] and *Lactococcus lactis* strain DPC3147 pMRC01 (44–79% identical [24] (Table 2). Based on these similarities with other Gram-positive Trs (or Tra) regions, this gene cluster is presumed to be responsible for conjugative functions associated with pLU4.

In addition to *trs* gene products, several CHPs were found in this region. A well-conserved peptidoglycan hydrolase (PGH, *orf15*) with a lysozyme-like domain (cd00254) and a cell-wall-associated hydrolase domain (Spr, COG0791) [25] was identified within the conjugation loci, as found in other conjugative plasmids [26]. PGH is required for efficient conjugative transfer of plasmids, presumably to facilitate penetration of the plasmid translocation complex through the peptidoglycan layer of cell walls [27]. A well-conserved thioredoxin (TRX)-like protein (ORF17) with a TRX fold and a redox active C-X-X-C motif [28] was also found in this region. A protein belonging to the disulfide bond formation family plays an essential role in the conjugative process by mediating folding and/or assembly of pore complex proteins [29], suggesting that ORF17 is also involved in the conjugation process. ORF3 was found to be highly conserved (100% identical) in 42 species of *Lactobacillaceae,* including

Table 1 Putative genes, domains, and their proposed functions deduced from 41 potential coding regions

Gene	Codon		No. of amino acids	Best homolog[b] [organism (no. of strains), GenBank accession no.]	% Identity (no. of amino acids overlapping)	Conserved domain (accession no.)	Proposed function
	Start	Stop[a]					
repA	1	1122	373	Rep protein [*L. gasseri*, WP_049160251.1]	99 (373)	RepA_N (pfam06970)	Plasmid replication
relB	1382	1663	93	DinJ [*Lactobacillaceae* multispecies (24), WP_003646131.1]	99 (93)	RelB_DinJ (TIGR02384)	Plasmid stabilization
orf3	1710	1895	61	CHP [*Lactobacillaceae* multispecies (42), WP_010620892]	100 (61)	–	–
orf4	1892	2572	226	ATPase [*Lactobacillus* multispecies (3), WP_007124583.1]	98 (226)	P-loop_NTPase (cl21455)	Conjugative transfer
orf5	2562	2840 C	92	CHP [*L. versmoldensis*, WP_010625587.1)	98 (92)	DUF3847 (cl15196)	–
orf6	2863	3090 C	75	CHP [*Lactobacillaceae* multispecies (6), WP_003555660.1]	95 (75)	–	–
orf7	3343	5406	687	Nickase, TraA-like [*L. plantarum*, WP_063733792.1]	99 (687)	MobA_MobL (pfam03389)	Conjugative transfer
orf8	5490	5801	103	CHP [*Lactobacillus* multispecies (7), WP_003712158.1]	100 (103)	–	–
orf9	5838	6452	204	CHP (*L. casei*, WP_012552809.1)	98 (204)	–	–
trsB	6454	6789	111	TraB protein [*Lactobacillus* multispecies (5), WP_003590767.1)	100 (111)	T4SS_CagC (pfam16943)	Conjugative transfer
trsC	6810	7172	120	TraC protein [*Lactobacillus* multispecies (15), WP_003590770.1]	100 (120)	T7_EssCb_Firm (TIGR03928)	Conjugative transfer
trsD	7141	7800	219	TraD protein [*Lactobacillus* multispecies (3), WP_003712179.1]	100 (219)	VirB4_CagE (TIGR00929)	Conjugative transfer
trsE	7812	9830	672	Protein TrsE [*L. paracasei*, WP_016370338.1]	99 (672)	CagE_TrbE_VirB (pfam03135), VirB4 (COG3451)	Conjugative transfer
trsF	9823	11241	472	TraF protein [*L. casei* WP_003590776.1]	97 (472)	T7_EssB (TIGR03926)	Conjugative transfer
orf15	11242	12399	385	Peptidoglycan hydrolase [*Lactobacillus* multispecies (2), WP_049170325.1]	99 (385)	Spr (COG0791)	Conjugative transfer
orf16	12413	13030	205	CHP [*L. oris*, WP_003712154.1]	98 (205)	GH18_chitinase-like (cl10447)	–
orf17	13017	13385	122	Thioredoxin [*Lactobacillus* multispecies (3), WP_016370342.1)	99 (122)	TRX_family (cd02947)	Conjugative transfer
trsJ	13386	13856	156	TraJ protein [*L. helveticus* (5), WP_003625649.1]	97 (156)	–	Conjugative transfer
orf19	13853	14089	78	HP [*L. pentosus* MP-10, CCB82908.1]	94 (78)	–	–
trsK	14086	15636	516	TraK protein [*L. paralimentarius*, WP_056955663.1)	99 (516)	TraG_VirD4 (cd01126)	Conjugative transfer
orf21	15651	16040	129	CHP [*Lactobacillus* multispecies (6), WP_003712166.1]	98 (129)	–	–
trsL	16059	16898	279	TraL protein [*L. gasseri*, WP_012621107.1]	99 (279)	–	Conjugative transfer

Table 1 continued

Gene	Codon		No. of amino acids	Best homolog[b] [organism (no. of strains), GenBank accession no.]	% Identity (no. of amino acids overlapping)	Conserved domain (accession no.)	Proposed function
	Start	Stop[a]					
orf23	16913	17323	136	CHP [*L. plantarum*, WP_016511108.1]	98 (136)	–	–
trsI	17330	19465	711	DNA topoisomerase III [*L. plantarum*, WP_062688604.1]	98 (711)	TOP1Ac (cd00186)	Conjugative transfer
orf25	19585	19800	71	CHP [*Lactobacillaceae* multispecies (17), WP_003713050.1]	99 (71)	–	–
ltrC	19803	20927	374	LtrC-like protein [*L. curvatus*, WP_039098912.1]	94 (374)	ArdC (COG4227)	Conjugative transfer
orf27	21127	21306	59	CHP LRH_03875 [*L. rhamnosus* HN001, ACH91653.1]	98 (59)	–	–
orf28	21637	22986	449	Pyridine nucleotide-disulfide oxidoreductase [*L. casei*, WP_012552825.1]	99 (449)	Pyr_redox (pfam00070) Pyr_redox_dim (pfam02852)	–
orf29	23139	23504	121	HNH endonuclease [*L. salivarius*, WP_047036278.1]	97 (305)[c]	HNHc (cd00085)	Phage DNA packaging
orf30	23517	24020	167	SMI1/KNR4 family protein [*Lactobacillus* multispecies (4), WP_011241750.1]	99 (167)	SMI1_KNR4 (pfam09346)	–
orf31	24312	27482 C	1056	Collagen-binding protein [*L. fermentum*, WP_048340564.1]	95 (1055)	Cna_B (pfam05738) CollagenBindB (cd00222)	Bacterial adhesion
orf32	27508	27648	46	HP LBGG_02325 [*L. gasseri* MV-22, EFQ45516.1]	93 (60)[c]	–	–
orf33	27775	27954 C	59	HP [*Lactobacillus* multispecies (1), WP_032954691.1]	100 (59)	–	–
orf34	28034	28618	194	α/β hydrolase [*L. oris* (2), WP_056984888.1]	99 (194)	Abhydrolase_5 (pfam12695)	–
orf35	28739	29323	194	Putative resolvase [*Lactobacillus* multispecies (3), WP_056984887.1]	100 (194)	Resolvase (pfam00239)	Recombination
orf36	29343	29525 C	60	CHP [*Lactobacillus* multispecies (4), WP_016370330.1]	100 (60)	–	–
orf37	29610	30062 C	150	CHP [*Lactobacillus* multispecies (6), WP_003590731.1]	100 (150)	–	–
orf38	30323	30793	156	HP [*L. paracasei* subsp. *Paracasei*, ABA12837.1]	100 (156)	–	–
orf39	31103	31273 C	56	DinJ damage inducible [*Lactobacillaceae* multispecies (9), WP_003590736.1]	100 (56)	RelB (pfam04221)	Plasmid stabilization
orf40	31754	32545	263	Peptide transporter [*Lactobacillus* multispecies (6), WP_003712521.1]	99 (263)	BcsQ (COG1192)	Plasmid replication
orf41	32617	32856	79	CHP [*Lactobacillus* multispecies (5), WP_016370331.1]	100 (79)	–	–

[a] *C* complementary sequence

[b] *CHP* conserved hypothetical protein, *HP* hypothetical protein

[c] Homologous to C-terminal part of protein

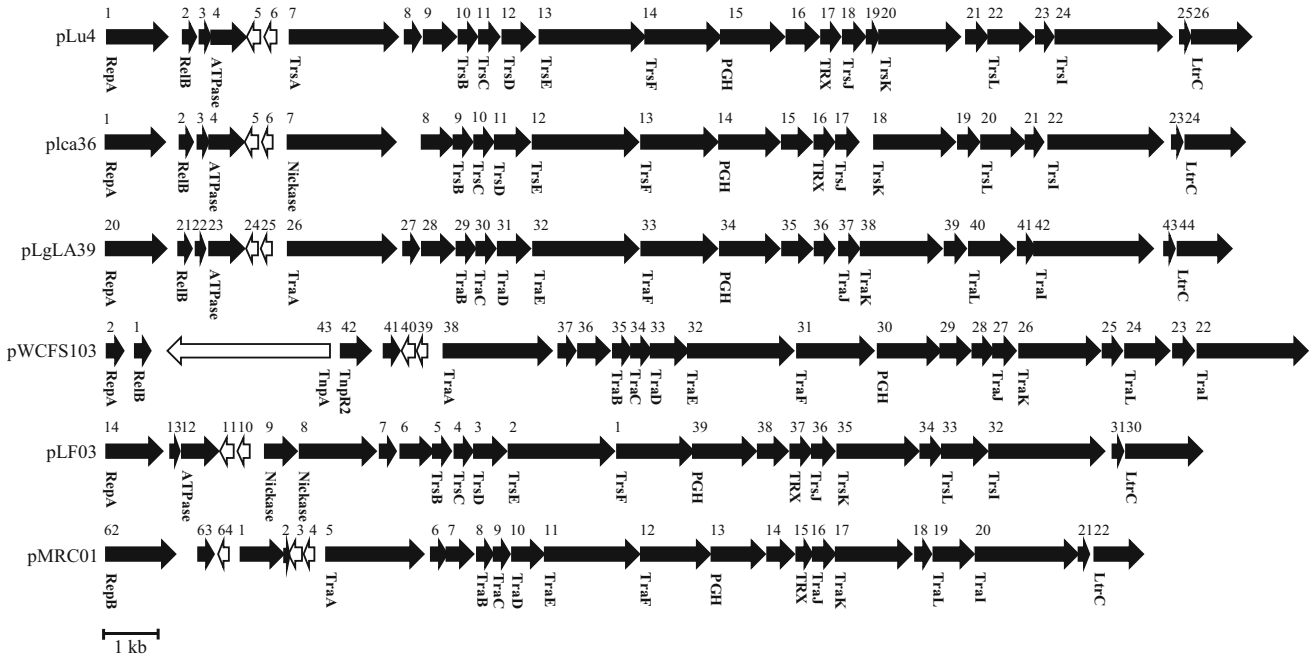

Fig. 2 Comparison of gene cluster regions for conjugative transfer between pLU4 [ORF1 (RepA) to ORF26 (LtrC)] and four conjugation plasmids from lactic acid bacteria. Numbers on *top* of each *arrow* (ORF) indicate arbitrary ORF numbers assigned to each plasmid. The accession number and bacterial strain of each plasmid used for comparative analyses are pLU4 (KM063576.1, *L. reuteri* strain LU4), plca36 (CP000935.1, *L. casei* str. Zhang), pLgLA39 (AB436615.1, *L. gasseri* LA39), pWCFS103 (CR377166.1, *L. plantarum* WCFS1), pLF03 (AVAB01000112.1, *L. fermentum* MTCC 8711), and pMRC01 (AE001272.1, *Lactococcus lactis* strain DPC3147)

plca36 and pLgLA39. A protein identical to ORF8 was also identified in seven *Lactobacillus* species, including pLgLA39, but not in plca36. However, their potential functions in conjugation are unknown because neither has a conserved domain. ORF19 is unique in pLU4, and its putative function is unknown.

Previously, Ito et al. [21] reported that *L. reuteri* LA6 harbored a plasmid (pLrLA6) almost identical to pLgLA39 in *L. gasseri*, based on a restriction enzyme digestion analysis. They also showed that pLrLA6 is reuterin- and conjugation-positive, indicating that pLrLA6 might possess genes for bacteriocin production and conjugation. Unlike pLrLA6, pLU4 does not carry a gene for an antibiotic, suggesting that these two species evolved in somewhat different ways. Because the pLrLA6 sequence has not yet been published, their similarities and differences could not be determined.

Genes for plasmid replication and maintenance of stability

ORF1 of pLU4 is a plasmid replication initiation protein (RepA) containing a pfam 06970 RepA_N domain at its N terminus. RepA is nearly identical (99% over 373 aa) to that of *L. gasseri* (WP_049160251.1) and is also similar to those of several *Lactobacillus* plasmids, including *L. gasseri* JCM 8787 pLgJCM8787 (BAH15378.1), *L. gasseri* OLL2935 pLgOLL2935 (BAH15382.1), *L. rhamnosus* HN001 pLR002 (ACH91646.1), plca36 (ACI34418.1), and pLgLA39 (BAH08724.1), with 85–91% identities over the entire amino acid sequence.

Stable inheritance or maintenance of bacterial plasmids is accomplished through different strategies, such as post-segregational killing systems or an addiction module [toxin-antitoxin (TA) system], multimer resolution systems, and plasmid partition systems [30, 31, 32]. The *relBE* module encodes the toxin RelE and upstream antitoxin RelB, which constitutes a TA system involved in plasmid maintenance [33]. The complete *relBE* locus in a *Lactobacillus* plasmid was reported only in plca36 from *L. casei* str. Zhang; this plasmid encodes a *relBE* locus (*orf40* and *orf41*) and an orphan *relB* locus (*orf2*) located downstream of RepA (*orf1*) [20]. In this study, two *relB* loci (*orf2* and *orf39*) were identified. Gene *orf2* was found to encode 93 amino acids of a putative DNA-damage-inducible protein (RelB antitoxin), with a theoretical molecular weight (MW) of 10.4 kDa and isoelectric point (pI) of 4.72. ORF2 is almost identical (99%) to DNA-damage-inducible protein J (DinJ) found in 24 *Lactobacillaceae* species (WP_003646131.1). ORF2 is also highly similar to RelB proteins in pWCFS103, pLgLA39, and plca36 plasmids (97, 96, and 86% identical, respectively). The RelB proteins encoded by the three plasmids are commonly downstream of *repA*, as it is in pLU4, suggesting that RelB

Table 2 Similarity (% of identity) of ORFs in the conjugative gene cluster between pLU4 and other lactic acid bacterial plasmids

Plasmids Proteins	plca36 (*L. casei* Zhang)	pLgLA39 (*L. gasseri*)	pWCFS103 (*L. plantarum*)	pLF03 (*L. fermentum*)	pMRC01 (*Lactococcus lactis*)
ORF3 (61)	100% (P3)	100% (P22)	–	57%	–
ATPase (226)	95% (ATPase)	86% (ATPase)	–	62% (ATPase)	–
ORF5 (92)	95% (P5)	97% (P24)	93% (P40)	60% (P11)	NH (64, P3)
ORF6 (75)	94% (69, P6)	94% (69, P25)	94% (69, P39)	53% (94, P10)	57% (94, P4)
TrsA (687)	97% (691, Nickase)	96% (TraA)	67% (686, TraA)	pseudogene	50% (680, TraA)
ORF8 (103)	–	100% (P27)	71% (112, P37)	59% (106, P7)	52% (114, P6)
ORF9 (204)	98% (P8)	95% (P28)	94% (P36)	62% (208, P6)	50% (201, P7)
TrsB (111)	99% (TrsB)	100% (TraB)	100% (TraB)	76% (114, TraB)	72% (114, TraB)
TrsC (120)	100% (TrsC)	100% (TraC)	98% (TraC)	80% (116, TraC)	72% (115, TraC)
TrsD (219)	99% (TrsD)	99% (TraD)	98% (TraD)	82% (217, TraD)	79% (217, TraD)
TrsE (672)	98% (TrsE)	97% (TraE)	97% (TraE)	86% (TraE)	84% (TraE)
TrsF (472)	97% (TrsF)	91% (TraF)	92% (TraF)	59% (TraF)	51% (TraF)
PGH (385)	98% (P14)	97% (PGH)	96% (384, PGH)	77% (384, PGH)	54% (PGH)
ORF16 (205)	93% (P15)	95% (P35)	92% (P29)	54% (P38)	48% (P14)
TRX (122)	89% (110, TRX)	90% (TRX)	91% (TRX)	60% (94, TRX)	43% (TRX)
TrsJ (156)	96% (TrsJ)	88% (TraJ)	88% (TraJ)	52% (152, TraJ)	50% (152, TraJ)
ORF19 (78)	–	–	–	–	–
TrsK (516)	97% (514, TrsK)	98% (518, TraK)	93% (503, TraK)	82% (514, TraK)	77% (530, TraK)
ORF21 (129)	98% (P19)	98% (135, P39)	43% (124, P25)	53% (131, P34)	45% (131, P18)
TrsL (279)	99% (TrsL)	99% (TraL)	96% (285, TraL)	43% (278, TraL)	44% (278, TraL)
ORF23 (136)	92% (P21)	92% (134, P41)	78% (P23)	–	–
TrsI (711)	96% (TrsI)	97% (712, TraI)	96% (TraI)	70% (722, TraI)	60% (725, TraI)
ORF25 (71)	55% (P23)	55% (P43)	55% (P21)	60% (72, P31)	58% (69, P21)
LtrC (374)	83% (LtrC)	87% (LtrC)	–	77% (469, LtrC)	58% (355, LtrC)

Number and word in parenthesis indicate total number of amino acids and arbitrary name of each protein, respectively

NH no homology, "–" means "no ORF or protein encoded"

might play similar roles in these plasmids. Gene *orf39* was found to encode 56 amino acids of a putative DinJ, with a theoretical MW of 6.3 kDa and pI of 9.87. A RelB domain (pfam04221) was identified (aa position 1–50), along with a similar RelB_DinJ domain (TIGR02384) (aa position 4 to 52). ORF39 is identical (100%) to DinJ proteins in nine *Lactobacillaceae* species (WP_003590736.1). Thus, RelB/DinJ homologs, ORF3 and ORF39, are well conserved in *Lactobacillus* species, suggesting that they are universal and have some other functions. Unlike to plca36, which has a typical *relBE* locus [20], and pLgLA39, which has a *pemIK*-like module of another TA system [21], no RelE homologs or other TA systems were identified in pLU4, indicating that pLU4 has an incomplete *relBE* module and does not encode a TA system.

Multimer resolution systems act by converting multimeric plasmids to monomers for proper plasmid partitioning. Site-specific recombination by resolvase contributes to the stable inheritance of plasmids and thereby increases the segregational stability of plasmids [31, 34]. ORF35 in pLU4 encodes a putative resolvase that contains an N-terminal catalytic domain (pfam00239) and a C-terminal DNA-binding domain including a helix-turn-helix motif (HTH_7, pfam02796). Identical resolvases were found in three *Lactobacillus* species (WP_056984887.1), and almost identical proteins also exist in plca36 (ORF37, 99% identical) and pLgLA39 (ORF12, 97% identical). This may suggest that this type of resolvase contributes to the maintenance of plasmid stability in *Lactobacillus* species.

Partition systems actively separate and distribute plasmid molecules to distal parts of the parental cell before it undergoes division, guaranteeing that the daughter cells inherit at least one copy of the plasmid [30]. The *parABS* system is a fairly conserved molecular mechanism for plasmid partitioning and chromosome segregation in Gram-positive bacteria. This system consists of three components: the ParA ATPase, the ParB DNA-binding protein, and the cis-acting *parS* sequence [35–37]. ORF40 in pLU4 is a peptide transporter encoding ParA (cd02042) domain at the N terminus (aa position 3–173). Almost

identical proteins (99%), named chromosome partitioning ATPase, have been found in several *Lactobacillus* species (WP_003712521.1). However, the *parB* gene and *parS* sequence, which comprise a complete *parABS* plasmid partition system, were not found in pLU4, suggesting that ORF40 might play other roles.

Other genes

Adhesion is believed to be essential for probiotic effects such as immunomodulation and competitive exclusion of pathogens in the gastrointestinal mucosa [38]. A 29-kDa collagen-binding protein (CBP) was previously isolated from *L. reuteri* NCIB 11951 [39] and is thought to regulate the binding of this strain to host tissues. Herein, a putative CBP was identified (ORF31, 1,056 aa), with a Can protein B-type domain (Can_B, pfam05738) and five 23-kDa repeat units of collagen-binding protein domain B (CollagenBindB, cd00222) at the C terminus. ORF31 is almost identical to a CBP (1,055 aa) of *L. fermentum* 3872 pLF3872 (95% identical, 1,009 out of 1,057 aa, [40] and a putative CBP (ORF32, 876 aa) of *L. casei* Zhang plca36 (95% identical, 762 out of 803 aa). However, compared to plca36 CBP, pLU4 ORF31 is unique because of the presence of two additional copies of the CollagenBinB repeat domain (ca. 86 aa in size), which may be responsible for binding to host cell receptors.

ORF34 is an alpha/beta hydrolase of 194 aa with a conserved motif, YdeN (COG3545), that is a predicted esterase of the alpha/beta hydrolase fold. This fold is commonly found in hydrolytic enzymes with diverse phylogenetic origins and catalytic functions [41]. A homology search showed that ORF34 is almost identical (99%) to a predicted esterase-like protein encoded in a large plasmid of the probiotic strain *L. paracasei* NFBC338 [42]. Because the alpha/beta hydrolase fold family is one of the largest groups of structurally related enzymes with diverse catalytic functions [43], the putative function of ORF34 protein remains to be solved.

In conclusion, the large plasmid pLU4, found in *L. reuteri* strain LU4, encodes 41 potential ORFs. Most of these ORFs showed over 95% identities with conserved proteins identified in plasmids and/or chromosomal DNAs of *Lactobacillus* species. The detailed genetic analysis revealed that pLU4 contains functional genes for conjugation and plasmid replication and maintenance of stability, as well as other conserved genes with cryptic roles. A gene cluster for conjugative transfer, including 10 *trs* genes in pLU4, showed very similar organization to gene clusters in several other lactic acid bacterial plasmids (seen in Fig. 2). Moreover, a similarity search of gene sequences in the pLU4 cluster region revealed the highest identity with either *L. casei* str. Zhang plca36 or *L. gasseri* LA39 pLgLA39, suggesting that these homologies might have resulted from plasmid recombination and horizontal transfer among *Lactobacillus* or evolution from common ancestors. A better understanding of the genetic and molecular basis of *Lactobacillus* plasmids would help alleviate concerns about the use of *Lactobacillus* species as probiotics and also allow factors associated with probiotic functions to be identified. In addition, this understanding could be used to develop probiotics in more effective ways, e.g., genetically engineered probiotics for therapeutic applications.

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