Conjugative Plasmid from *Lactobacillus gasseri* LA39 That Carries Genes for Production of and Immunity to the Circular Bacteriocin Gassericin A[▽]

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Gassericin A is a circular bacteriocin produced by *Lactobacillus gasseri* strain LA39. We found a 33,333-bp plasmid, designated pLgLA39, in this strain. pLgLA39 contained 44 open reading frames, including seven genes related to gassericin A production/immunity (*gaa*), as well as genes for replication, plasmid maintenance, and conjugative transfer. pLgLA39 was transferred from LA39 to the type strain of *L. gasseri* (JCM 1131) by filter mating. The transconjugant exhibited >30-fold-higher more resistance to gassericin A and produced antibacterial activity. *Lactobacillus reuteri* LA6, the producer of reutericin 6, was proved to harbor a plasmid indistinguishable from pLgLA39 and carrying seven genes 100% identical to *gaa*. This suggests that pLgLA39 might have been transferred naturally between *L. gasseri* LA39 and *L. reuteri* LA6. The seven *gaa* genes of pLgLA39 were cloned into a plasmid vector to construct pGAA. JCM 1131[T] transformed with pGAA expressed antibacterial activity and resistance to gassericin A. pGAA was segregationally more stable than a pGAA derivative plasmid from which *gaaA* was deleted and even was more stable than the vector. This suggests the occurrence of postsegregational host killing by the *gaa* genes. pLgLA39 carried a *pemIK* homolog, and segregational stabilization of a plasmid by the pLgLA39-type *pemIK* genes was also confirmed. Thus, pLgLA39 was proved to carry the genes for at least two plasmid maintenance mechanisms, i.e., *gaa* and *pemIK*. Plasmids containing a *repA* gene similar to pLgLA39 *repA* were distributed in several *L. gasseri* strains.

Lactobacillus species are normal inhabitants of the human gastrointestinal tract, and *Lactobacillus gasseri* is one of the most commonly detected of these species (37, 47). Health-promoting effects of this species, such as immunomodulation (35), suppression of *Helicobacter pylori*-induced interleukin-8 production (44), and improvement of intestinal conditions (34), have been reported, and some *L. gasseri* strains are used in commercial probiotic products.

Bacteriocins are antimicrobial peptides, proteins, or protein complexes produced by bacteria and active mainly against related bacterial species (38). Several bacteriocins also inhibit the growth of food-borne pathogens, such as *Listeria*, *Bacillus cereus*, and *Clostridium perfringens*. Production of bacteriocin is thought to be a desired feature for probiotic strains, since bacteriocin is believed to provide an advantage for survival in the ecological niche and to prevent the growth of pathogens. Several *L. gasseri* strains are known to produce bacteriocins (18). The classification of bacteriocins remains controversial. We use the definition proposed by Maqueda et al. (30), where bacteriocins are classified into class I (lantibiotics), class II (nonlantibiotics), class III (large heat-labile bacteriocins), and class IV (circular bacteriocins linked at the N- and C-terminal ends). Among these, the class IV circular bacteriocins have attracted increasing attention, since they are the simplest pro-

karyotic representatives of the ubiquitous circular peptides with various physiological activities (6). Enterocin AS-48 from *Enterococcus faecalis* strain S-48 is the first and most vigorously characterized member of the class IV bacteriocins (30). *L. gasseri* strain LA39 (JCM 11657) produces a 58-amino-acid (aa) circular bacteriocin, gassericin A (18). Gassericin A is a representative of the non-AS-48-like circular bacteriocin group including butyrivibriocin AR10 from *Butyrivibrio fibrisolvens* AR10 (15) and carnocyclin A from *Carnobacterium maltaromaticum* UAL307 (32), as well as reutericin 6 from *Lactobacillus reuteri* LA6 (17) and acidocin B from *Lactobacillus acidophilus* M46 (26). The last two bacteriocins have nearly identical amino acid sequences to that of gassericin A. Though the number of reported circular bacteriocins has been increasing, their primary sequences and the genes responsible for production of and immunity to them are diversified (for a review, see reference 31). Recently, we isolated and sequenced seven genes (*gaaBCADITE*) from LA39 deduced to be responsible for production of and immunity to gassericin A (20). The *gaa* genes add new information to the complex world of the class IV bacteriocin genes.

The structural gene of gassericin A, *gaaA*, was reported to be located on the chromosome of LA39 (19). However, the high amino acid sequence identity of gassericin A to reutericin 6 (100%) and to acidocin B (98%) suggests recent horizontal gene transfers of the relevant bacteriocin genes, possibly via mobile elements. In fact, the acidocin B genes were reported to be located on a plasmid, namely, pCV461 (26). Many *Lactobacillus* strains are known to harbor one or more plasmids of various sizes, and several *Lactobacillus* plasmids have been

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[b]	Reference or source
Strains		
Lactobacillus gasseri strains		
LA39	Producer of gassericin A; isolated from feces of a human child; also known as JCM 11657	18
JCM 1131[T]	Type strain; also known as ATCC 33323	JCM[a]
JCM 1131[T](pSYE2)	Electrotransformant of JCM 1131[T] with pSYE2	This study
JCM 1131[T](pLgLA39/pSYE2)	pLgLA39 conjugant of JCM 1131[T](pSYE2)	This study
JCM 1131[T](pLgLA39)	pLgLA39 conjugant of JCM 1131[T] obtained from JCM 1131[T](pLgLA39/pSYE2) by curing pSYE2	This study
JCM 1131[T](pLrLA6)	pLrLA6 conjugant of JCM 1131[T] obtained from JCM 1131[T](pLrLA6/pSYE2) by curing pSYE2	This study
JCM 1131[T](pGAA)	Electrotransformant of JCM 1131[T] with pGAA	This study
JCM 1131[T](pGAAΔgaaA)	Electrotransformant of JCM 1131[T] with pGAAΔgaaA	This study
JCM 1131[T](pSYE2+T)	Electrotransformant of JCM 1131[T] with pSYE2+T	This study
JCM 1131[T](pPemIK)	Electrotransformant of JCM 1131[T] with pPemIK	This study
JCM 1131[T](pPemI)	Electrotransformant of JCM 1131[T] with pPemI	This study
JCM 1130	Also known as ATCC 19992	JCM
JCM 5344	Also known as ATCC 9857	JCM
JCM 8787		JCM
OLL2728, OLL2804, OLL2842, OLL2935, OLL2970	Laboratory collection of Meiji Dairies Corp.; isolated from human feces	This study
Lactobacillus reuteri LA6	Producer of reutericin 6	17
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842[T](pX3)	Type strain transformed with pX3; used as an indicator for bacteriocin activity	This study
Lactococcus lactis subsp. *lactis* IL1403	Plasmid-free; used as a cloning host in construction of plasmids	4
Plasmids		
pLgLA39	Isolated from *L. gasseri* LA39; Gaa⁺; conjugation positive	This study
pLrLA6	Isolated from *L. reuteri* LA6; Reu⁺; conjugation positive	This study
pSYE2	Cloning vector consisting of lactococcal plasmid pSY1 and *ermA*; Em^r	42
pGAA	Contains seven *gaa* genes (ORF04 to ORF10) of pLgLA39 (nt 1624 to 5878) cloned into the SmaI site of pSYE2; Gaa⁺ Em^r	This study
pGAAΔgaaA	Derivative of pGAA with *gaaA* deleted (see the text); Gaa⁻ Em^r	This study
pSYE2+T	Derivative of pSYE2 with the *Streptococcus thermophilus ldh* terminator and *Escherichia coli rrnBT1T2*; Em^r	This study
pPemIK	Derivative of pSYE2+T containing *pemIK*; Em^r	This study
pPemI	Derivative of pSYE2+T containing *pemI*; Em^r	This study
pX3	Derivative of *Lactobacillus delbrueckii* subsp. *bulgaricus* plasmid pBUL1 containing *ermA*; Em^r	36

[a] Japan Collection of Microorganisms.
[b] Gaa, gassericin A production; Reu, reutericin 6 production; Em^r, resistant to erythromycin.

reported to contain genes for production of bacteriocins (48). To our knowledge, however, only three have been sequenced entirely: these are pLA103 from *Lactobacillus acidophilus* TK8912 (16), pRC18 from *Lactobacillus curvatus* (previously known as *Lactobacillus casei*) CRL705 (7), and pMP118 from *Lactobacillus salivarius* subsp. *salivarius* UCC118 (5). Thus, genetic information about bacteriocin-producing *Lactobacillus* plasmids is still limited. Furthermore, little has been known about plasmids of *L. gasseri*, even though the existence of plasmids in a few strains has been reported, including a 26.5-kb anonymous plasmid in strain ADH (27) and pK7 in strain K7 (28).

Here we describe a 33.3-kb plasmid, designated pLgLA39, from *L. gasseri* LA39. The *gaa* genes are located on this plasmid. pLgLA39 carries a set of genes for conjugative transfer and was shown to be transmitted to another *L. gasseri* strain. *L. reuteri* LA6 also harbors a plasmid almost identical to pLgLA39. We demonstrated that production of gassericin A increased the apparent segregational stability of a plasmid carrying the *gaa* genes. A *pemIK* homolog in pLgLA39 was also

functional as a plasmid-stabilizing mechanism. This is the first report describing the entire nucleotide sequence and detailed genetic analysis of an *L. gasseri* plasmid, which contains functional genes for circular bacteriocin production, conjugation, and plasmid maintenance.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. *Lactobacillus* strains were grown statically in MRS medium (Becton Dickinson and Company, Sparks, MD) at 37°C. *Lactococcus lactis* subsp. *lactis* IL1403 (4) was grown in M17 medium (Becton Dickinson and Company) supplemented with 0.5% (wt/vol) glucose at 32°C. For plating, the media were solidified with 1.5% (wt/vol) agar. MRS-agar plates for *Lactobacillus* strains were incubated anaerobically by using an Anaeropack system (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan).

Electroporation. *L. gasseri* JCM 1131[T] was cultivated overnight at 37°C in modified MRS medium supplemented with 1% (wt/vol) glycine and adjusted to pH 4.5 with HCl, and preparation of the electrocompetent cells and electroporation were done by the procedure originally described for *Lactobacillus delbrueckii* subsp. *bulgaricus* (43). *L. lactis* IL1403 was electroporated according to the method of Holo and Nes (13). Transformants were selected by using 25 μg/ml erythromycin (Em).

TABLE 2. Primers used

Primer name	Sequence (5′ to 3′)[a]	Location (nt) or description[b]	Reference
pr01	ATGGTTACTAAGTACGGACG	2717–2736	
pr02	CTAGGCTGCAGTCGCTCCCA	2992–2973	
pr03	CCGCAGATAGGCTAGGTGTC	1624–1643	
pr04	TAATTCCTAGTATAGCACAG	5883–5864	
pr05	AAAACTCGAGAGTATCTTCCTCCCATAGG	AAAA plus XhoI site plus nt 2721 to 2703	
pr06	AAAACTCGAGTGATTATAAAGCTTTATGAATAG	AAAA plus XhoI site plus nt 2998 to 3020	
pr07	CCTCGCAAGGGAAAAGACAG	278–297	
pr08	GCAGAGCAACTGCTGGCGAG	1268–1249	
pr09	CTTCGTAACAATGCTATGAC	718–699	
pr10	ATGACAAAATCAACAGATTTC	12632–12652	
pr11	TTTAAAAGCCCCTATTCTTC	13841–13822	
pr_q01	CAGCTATGGGAAACGACTATGAA	6164–6186 (ATCC 33323 genome sequence)	29
pr_q02	CGTATTCTGTGATCTTGAGGTTG	6382–6360 (ATCC 33323 genome sequence)	29
pr_q03	CAACAACTGGGTTGATGAAGAAG	12799–12821	
pr_q04	AAATAGACATCGGTTGCTTTCAC	13026–13004	
pr_q05	TACCTTGGATATTCACCGAACAC	501–479 (pSYE2)	42
pr_q06	GTTGACGATATTCTCGATTGACC	287–309 (pSYE2)	42
27F	AGAGTTTGATC(A/C)TGGCTCAG	Used to amplify 16S rRNA gene	25
1492R	TACGG(C/T)TACCTTGTTACGACTT	Used to amplify 16S rRNA gene	25

[a] The XhoI site is underlined.
[b] Numbers indicate coordinates in the nucleotide sequence of pLgLA39, unless otherwise indicated.

DNA isolation and manipulation. Plasmid and total DNAs were prepared from *L. gasseri* cells after limited cell wall hydrolysis. *L. gasseri* cells were incubated at 37°C for 10 min in PBE (0.3 M raffinose, 1 mM EDTA, 20 mM Tris, pH 7.0) containing 2 mg/ml lysozyme and 0.04 mg/ml mutanolysin (Sigma-Aldrich, St. Louis, MO). Afterward, plasmid DNA was prepared by the standard alkaline sodium dodecyl sulfate method (40). To prepare the total DNA, *L. gasseri* cells, partially lysed as described above, were suspended in TE (1 mM EDTA, 10 mM Tris, pH 8.0) and thoroughly lysed. The total DNA was purified from the lysate by phenol extraction and ethanol precipitation (40). Further purification was done with standard RNase A treatment and polyethylene glycol precipitation (40). Similar methods were used to isolate plasmid and total DNAs from *L. reuteri* LA6 and plasmid DNA from *L. lactis*, except for using 6.7% (wt/vol) sucrose, 1 mM EDTA, and 50 mM Tris (pH 8.0) in place of PBE in the limited cell wall hydrolysis step.

DNA manipulation was done according to standard methods (40) or instructions from suppliers. PCRs were performed on a PerkinElmer model 9700 thermal cycler with *Ex Taq* polymerase (Takara Bio Inc., Otsu, Japan) unless otherwise stated. Primers used in PCRs are listed in Table 2. All oligonucleotides were purchased from Operon Biotechnologies (Tokyo, Japan). *gaaA* and the 16S rRNA gene were amplified from the total DNA of LA39 by using primer sets pr01-pr02 and 27F-1492R (25), respectively, and were labeled with an AlkPhos Direct kit (GE Healthcare UK Ltd., Buckinghamshire, England) and used as probes in Southern hybridization. Detection was done using a CDPStar detection kit (GE Healthcare UK Ltd.). For Fig. 3B, restriction fragments were analyzed on a DNA 7500 LabChip, using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Sequence determination and genetic analysis of pLgLA39. The 4,100-bp sequence containing the seven *gaa* genes was determined previously (20). First, sequences were extended in both directions by primer walking, using the total DNA of LA39 as a template. After identification of pLgLA39 and localization of the *gaa* genes on pLgLA39 (Fig. 1), several primers based on the sequences of other *L. gasseri* plasmids we had sequenced (unpublished results) and on *Lactobacillus paracasei* subsp. *paracasei* contig 2 (8) were used to amplify partial fragments of pLgLA39. The resulting amplicons were sequenced by primer walking and assembled to give pLgLA39, a circular plasmid of 33,333 nucleotides (nt). Sequencing was performed using dye terminator sequencing reagents and an ABI3100 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan). Sequence analyses were done with Genetyx software (Genetyx Corp., Tokyo, Japan). Open reading frames (ORFs) in pLgLA39 were predicted by the GeneMark.hmm program (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prok.cgi), using the *Lactobacillus johnsonii* NCC 533 genome as a reference. Amino acid sequences of the deduced ORFs were subjected to homology searches against the NCBI nonredundant database by using the BLAST program (2) and were functionally categorized by using the COG database (45). Sequence alignments were done by using the ClustalW2 web service (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Enrichment and measurement of gassericin A activity. Bacteriocin activity was assayed using the stepwise dilution method. *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (type strain) transformed with pX3 (36) was used as the standard indicator strain in this study. An arbitrary unit (AU) of activity was defined as the reciprocal of the highest dilution that inhibited the growth of the indicator strain. Since pX3 conferred Em resistance on ATCC 11842[T], culture supernatants containing Em were assayed using the indicator ATCC 11842[T](pX3) without removing the antibiotics.

A 20× concentrate of LA39 culture supernatant was prepared as follows. Ammonium sulfate (30% [wt/vol]) was added to the overnight culture supernatant of LA39, and the precipitate was dissolved in 20 mM sodium phosphate buffer (pH 5.0) at 1/20 the volume of the initial supernatant. The activity of the concentrate was usually 3,200 to 20,480 AU per ml.
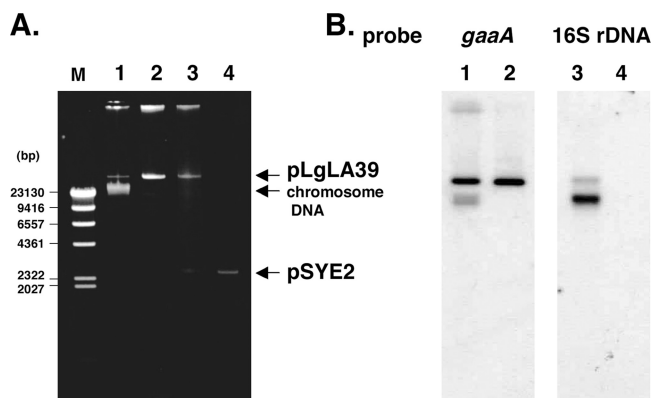

FIG. 1. Confirmation of pLgLA39. (A) Agarose gel electrophoresis. Lanes: M, molecular size marker (λ HindIII digest); 1, total DNA preparation from LA39; 2, purified pLgLA39 plasmid; 3, plasmid preparation from JCM 1131[T](pLgLA39/pSYE2); 4, pSYE2. (B) Southern hybridization. Lanes: 1 and 3, total DNA preparation from LA39; 2 and 4, purified pLgLA39 plasmid. The probes used were the *gaaA* probe (see Fig. 2) for lanes 1 and 2 and the 16S rRNA gene probe for lanes 3 and 4.

Conjugation. Conjugation was performed using the filter mating method described for *Lactobacillus plantarum* (41). Cells from overnight cultures of the donor (LA39) and the recipient (JCM 1131^T harboring pSYE2 [42]) were mixed at a ratio of 10:1, 1:1, or 1:10, concentrated by vacuum on a sterile membrane filter (pore size, 0.45 μm) (HA-type filter; Millipore Corp., Billerica, MA), and washed with 20 ml of 20 mM Tris (pH 7.0). The membrane filter was placed on an MRS plate and incubated at 37°C overnight. The cells on the filter were suspended in 3 ml of MRS medium, diluted stepwise in sterile saline, and plated. Transconjugants were selected on MRS plates containing gassericin A (the 20× concentrate of LA39 culture supernatant at 5% [vol/vol]) and 25 μg/ml Em. CFU on MRS plates (donor and recipient cells) and on MRS plates containing Em (recipient cells) were counted to calculate conjugation frequencies for donor cells and for recipient cells. Curing of pSYE2 from the conjugants was done by repetitively culturing the conjugant cells in Em-free MRS medium. The resulting Em-sensitive colonies were checked for the absence of pSYE2 in the plasmid preparation.

Construction of plasmids. To construct pGAA (see Fig. 4A), the seven *gaa* genes were amplified from pLgLA39 by a PCR using primers pr03 and pr04 and cloned into the SmaI site of pSYE2. To delete *gaaA* from pGAA, an inverse PCR was done on pGAA, using primers pr05 and pr06 (see Fig. 4A), and the amplicon was digested with XhoI and recircularized. The *gaaA* deletant plasmid was designated pGAAΔgaaA (see Fig. 4A). pGAAΔgaaA was completely sequenced to confirm that it did not contain any mutations due to PCR. pSYE2+T (see Fig. 5A) was constructed from pSYE2 by cloning the *Streptococcus thermophilus ldh* terminator (14) and the *Escherichia coli rrnBT1T2* terminator from pKK223-3 (GE Healthcare UK Ltd.). We had sequenced another *L. gasseri* plasmid that carries *pemIK* genes that are 99.7% identical to those of pLgLA39 (unpublished results). *pemIK* and *pemI* were amplified from the plasmid by using primer sets pr07-pr08 and pr07-pr09, respectively. These fragments were inserted into the SmaI site of pSYE2+T to obtain pPemIK and pPemI, respectively (see Fig. 5A). All plasmid constructions were done in *L. lactis* IL1403 as a host.

Segregational stability of plasmids. *L. gasseri* JCM 1131^T transformants harboring each plasmid were inoculated at 0.1% (vol/vol) into 1 ml MRS medium and grown. This cultivation, which corresponds to approximately 10 generations, was repeated, and the cultures were spread on MRS plates after the indicated number of times of repeated cultivation. One hundred colonies from each culture were checked for resistance to Em, indicating the presence of the plasmids. The experiments were done in duplicate.

Copy number determination. Copy numbers of the plasmids were determined by quantitative real-time PCR (qPCR). qPCR was executed using a QuantiTect SYBR green PCR kit (Qiagen KK, Tokyo, Japan) on a Rotor-Gene RG-3000 thermal cycler (Corbett Research, Sidney, Australia) under the following conditions: initial denaturation at 95°C for 15 min; 45 cycles of denaturation (94°C for 15 s), annealing (55°C for 30 s), and extension (72°C for 20 s); and final incubation at 72°C for 60 s and 60°C for 40 s. To determine the copy number of pLgLA39 in LA39, a 219-bp fragment of *gyrB* on the chromosome and a 228-bp fragment of pLgLA39 *repA* (ORF20) were amplified from the total DNA of LA39, using primer sets pr_q01-pr_q02 and pr_q03-pr_q04, respectively. The copy numbers of pGAA, pGAAΔgaaA, pSYE2, pPemIK, pPemI, and pSYE2+T in *L. gasseri* JCM 1131^T were determined similarly, by amplifying a 215-bp fragment of *ermA*, using primers pr_q05 and pr_q06, and the *gyrB* fragment from the total DNAs of the respective clones. The copy number relative to the chromosome equivalent was calculated from the ratio of the quantity of the *repA* or *ermA* amplicon to that of the *gyrB* amplicon. All of the experiments were done for two independent clones.

Nucleotide sequence accession numbers. The nucleotide sequence of pLgLA39 was submitted to DDBJ and given the accession number AB436615. The accession numbers for the *repA* sequences of the plasmids from the eight *L. gasseri* strains are AB478699 to AB478706.

RESULTS

Genes for gassericin A production (*gaa*) are located on a native plasmid of *L. gasseri* LA39. The seven *gaa* genes were identified by primer walking from the structural gene of gassericin A, *gaaA* (20). *gaaA* was reported to be located on the chromosome (19, 20). However, since we had revealed the presence of plasmids in several *L. gasseri* strains and had determined the nucleotide sequences of some of the plasmids (unpublished results), we reinvestigated the possibility that the

gaa genes might be located on an as yet unidentified plasmid of *L. gasseri* LA39. Careful inspection of plasmid content in LA39 revealed the presence of a large plasmid (Fig. 1A). The *gaaA* probe (Fig. 2) hybridized to the plasmid (Fig. 1B). This indicated that *gaaA* and the other *gaa* genes were located on the plasmid, not on the chromosome. The plasmid was designated pLgLA39. The copy number of pLgLA39 in LA39 was estimated to be 21.5 ± 0.7 (average for two clones ± standard deviation) per chromosome equivalent.

Sequencing of pLgLA39. We had previously found plasmids in several *L. gasseri* strains (see below) and had determined the entire sequences of two plasmids (unpublished results). pLgLA39 contained several regions substantially identical to these *L. gasseri* plasmids (unpublished results). Furthermore, pLgLA39 revealed the presence of genes highly homologous to *tra* genes in contig 2 of an unnamed plasmid of *L. paracasei* (8). Accordingly, several primers based on the nucleotide sequences of these plasmids were used in sequencing of pLgLA39. The determined entire sequence of pLgLA39 was 33,333 bp long, with a GC content of 39.5%, which was slightly higher than that (35.3%) of the genome sequence of *L. gasseri* ATCC 33323 (3). The first nucleotide of the unique SphI site was set to the start of the pLgLA39 sequence.

Analysis of genes in pLgLA39. Forty-four ORFs of more than 100 bp were identified in pLgLA39. Amino acid sequences of the ORFs were subjected to homology searches against the NCBI nonredundant database and the COG database to speculate their functions. The results are shown in Table 3. Thirty-five ORFs of pLgLA39 were homologous (threshold E value, 1×10^{-5}) to proteins with a known or predicted function(s), six were homologous to hypothetical proteins with no proposed functions, and three (ORF04, ORF08, and ORF19) were unique to pLgLA39. Analysis by RBSfinder predicted a ribosome binding site-like sequence 6 to 12 nt upstream of each ORF, except for ORF03, ORF05, ORF11, ORF13, ORF14, ORF17, ORF23, and ORF24. Potential ρ-independent terminators were identified between ORF03 and ORF04, ORF06 and ORF07, ORF10 and ORF11, ORF14 and ORF15, ORF17 and ORF18, ORF19 and ORF20, ORF23 and ORF24, and ORF29 and ORF30 (Fig. 2) by using the TransTermHP program (22). The sections below give a detailed description of the pLgLA39 ORFs, grouped by confirmed or predicted functions.

***gaa* genes.** ORF04 to ORF10 of pLgLA39 correspond to the *gaa* genes, *gaaBCADITE* (20) (Table 3). Functional cloning of the seven genes and confirmation of these genes as responsible for production of gassericin A are described below. ORF06 (*gaaA*) is the structural gene of gassericin A, and ORF08 (*gaaI*) is the immunity determinant gene. GaaT (ORF09) and GaaE (ORF10) are possible transporters of gassericin A. GaaB (ORF04), GaaC (ORF05), and GaaD (ORF07) were supposed to be membrane associated, but their roles in gassericin A production/immunity remain unknown.

Replication and maintenance genes. ORF20 is the replication protein gene (*repA*) of pLgLA39. RepA encoded by pLgLA39 contains the pfam06970 RepA_N domain in its N terminus and constitutes a distinct phylogenetic branch, together with the RepA proteins from several *Lactobacillus* and *Lactococcus* plasmids. RepA from pLgLA39 is nearly identical
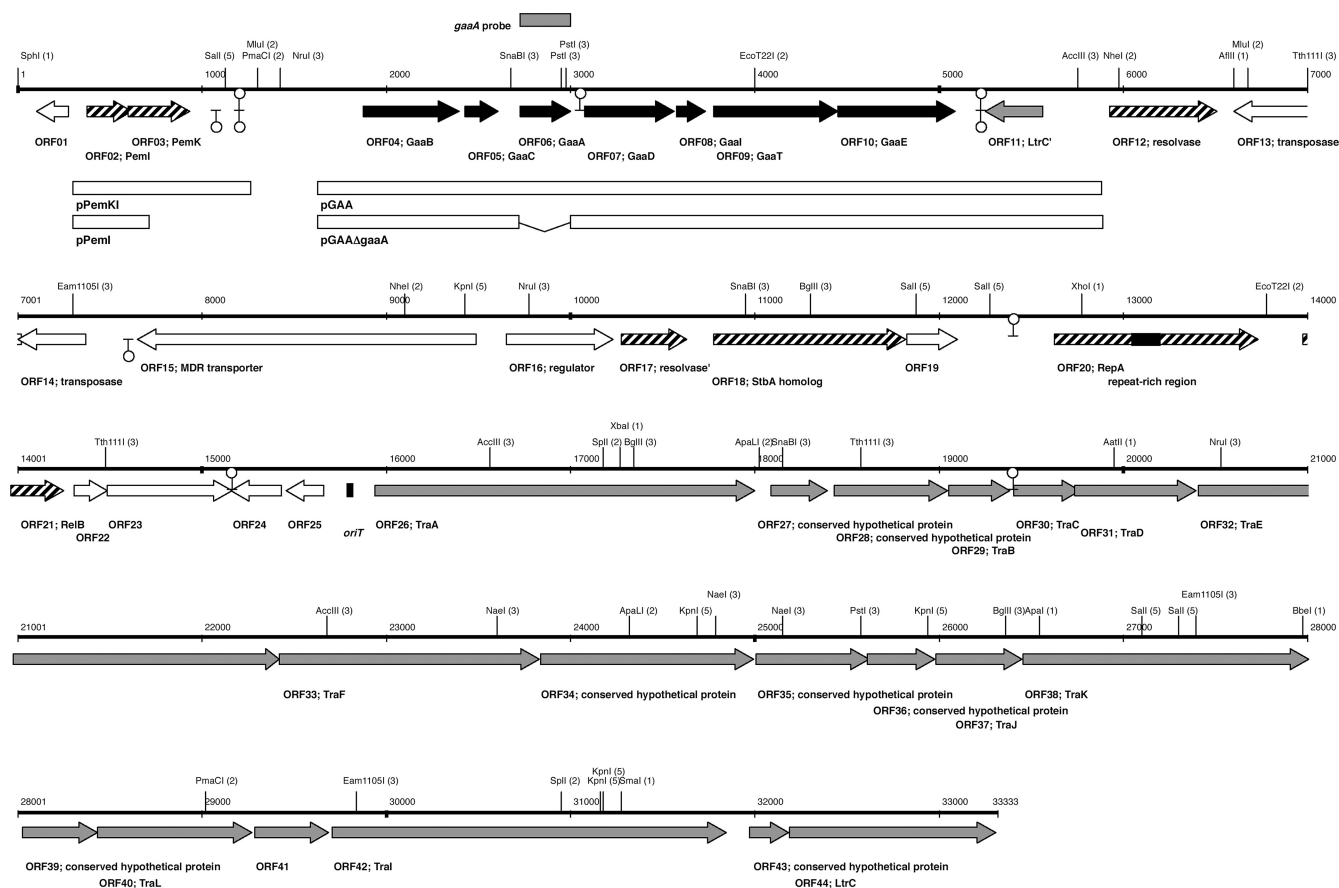
FIG. 2. Physical and genetic map of pLgLA39. Genes for gassericin A production, for conjugation, and for replication and maintenance are shown as black, shaded, and hatched arrows, respectively. Open and shaded boxes indicate the DNA regions used for construction of the plasmids and those used as the hybridization probe, respectively. A closed box indicates the supposed *oriT* sequence. The repeat region in *repA* is also indicated. Predicted terminators are shown by Ω-like symbols (upside-down ones are to terminate leftward transcriptions). Sites for rare-cutting restriction endonucleases are shown with total site numbers in parentheses.

(96% over 364 aa) to that from *L. salivarius* pSF118-44 (10) and also similar to those from several *Lactobacillus* and *Lactococcus* plasmids, such as *Lactobacillus brevis* ATCC 367 plasmid 1 (29), *L. salivarius* pSF118-20 (10), *L. lactis* pNP40 (33), *L. casei* ATCC 334 plasmid 1 (29), *L. lactis* pCI2000 (21), and *Lactobacillus sakei* pSAK1 (29), with 73.1% to 52.6% identities over the entire amino acid sequence. pLgLA39 and this group of plasmids likely replicate via the θ mechanism, since pSF118-20 was reported to be θ replicating (10). pLgLA39 *repA* contains a long direct repeat region (black box over *repA* in Fig. 2; nt 422 to 580 of *repA*) consisting of perfect repeats of 46 nt (5′-CTCAAACCCTTGCTACAAGCGGAAATGTGA AAATCACACTTCCGCA-3′) separated by 8 nt, with an additional 39-nt stretch identical to the 5′ terminus of the 46-nt sequence located 20 nt downstream. These nucleotide repeats result in three repeats of 12 aa (QTLATSGNVKIT) in the amino acid sequence of RepA. Such long repeats in the nucleotide sequence and amino acid repeats in the translation are common in *repA* genes of the related plasmids shown above. The 46-nt stretch contains a 12-nt inverted repeat near the 3′ terminus (underlined in the above sequence). This feature is also conserved in most of the *repA* sequences of the related plasmids, except for those of pCI2000 and pSAK1.

ORF02 and ORF03 encode proteins similar to several PemI-like and PemK-like proteins, respectively, encoded on chromosomes and plasmids of various bacterial genera. pLgLA39-encoded PemI and PemK showed the highest amino acid sequence identities to SMU.172 (79.0%) and SMU.173 (84.5%), respectively, of *Streptococcus mutans* UA159 (1). pLgLA39-encoded PemK showed 31.5% aa identity to the original PemK protein of the *E. coli* R100 plasmid, although pLgLA39-encoded PemI revealed little homology to R100-encoded PemI. *pemIK* of R100 is known as a toxin-antitoxin (TA) system (49). The functionality of the pLgLA39-type *pemIK* system was confirmed experimentally (see below). ORF03 (*pemK*) lacks a ribosome binding site, and the 5′ end of ORF03 partially overlaps the 3′ end of ORF02 (*pemI*). This suggests that expression of *pemK* may be regulated by translational coupling to *pemI* to strictly control the ratio of PemK to PemI, as observed with other TA systems (11).

ORF12 encodes a putative resolvase that contains a cd03768 SR_ResInv domain (serine recombinase family catalytic domain) in the N terminus and a cd00569 HTH_Hin-like (helix-turn-helix domain of Hin and related proteins) DNA-binding motif in the C terminus. Resolvases monomerize concatemeric plasmid molecules and thereby increase the segregational sta-

TABLE 3. Characteristics of ORFs and predicted proteins in pLgLA39

ORF	Location (nt)	Deduced function	Protein length (aa)	pI	Mol wt	COG	Cellular location[a]
01	269–93	Hypothetical protein	58	7.94	6,658.7		Soluble
02	366–611	PemI (inhibitor)	81	5.43	9,250.5	COG2336	Soluble
03	605–937	PemK (RNase toxin)	110	9.58	12,564.7	COG2337	Soluble
04	1871–2395	GaaB	174	9.60	20,278.4		Membrane (5)
05	2433–2615	GaaC	60	9.61	7,290.9		Membrane (2)
06	2722–2997	GaaA (gassericin A)	91	9.40	9,286.0	COG1300	Membrane (2)
07	3081–3569	GaaD	162	6.94	18,344.2		Membrane (4)
08	3591–3752	GaaI (immunity)	53	11.10	6,134.5		Membrane (1)
09	3775–4455	GaaT (transporter)	226	4.71	25,126.0	COG1131	Soluble
10	4458–5096	GaaE (transporter)	212	9.51	23,665.0		Membrane (6)
11	5560–5246	LtrC (fragment)	104	9.87	12,323.9		Soluble
12	5923–6507	Resolvase	194	9.99	22,341.8	COG1961	Soluble
13	7030–6599	Transposase (fragment)	143	8.75	16,163.5	COG3293	Membrane (1)
14	7374–7003	Transposase (fragment)	123	9.47	14,365.4	COG3293	Soluble
15	9491–7650	Multidrug resistance transporter	613	9.97	65,539.8	COG0477	Membrane (4)
16	9656–10240	Regulator	194	6.07	22,906.3	COG1309	Soluble
17	10325–10633	Resolvase (fragment)	102	10.22	12,215.2	COG1961	Soluble
18	10765–11814	StbA homolog	349	5.16	39,098.0	COG0443	Soluble
19	11817–12092	Hypothetical protein	91	5.82	10,228.7		Soluble
20	12632–13741	RepA	369	6.16	42,999.6		Soluble
21	13984–14265	RelB antitoxin	93	4.66	10,483.9	COG3077	Soluble
22	14312–14497	Conserved hypothetical protein	61	5.29	7,438.5		Soluble
23	14494–15174	Conserved hypothetical protein	226	9.17	25,968.5		Soluble
24	15442–15164	Conserved hypothetical protein	92	9.94	11,146.7		Soluble
25	15674–15465	Conserved hypothetical protein	69	9.30	7,971.1		Soluble
26	15946–18009	TraA (nicking enzyme)	687	8.92	80,624.2		Soluble
27	18094–18405	Conserved hypothetical protein	103	9.63	11,687.6		Soluble
28	18442–19056	Conserved hypothetical protein	204	5.11	23,696.4		Soluble
29	19058–19393	TraB	111	9.21	11,717.0		Membrane (3)
30	19414–19776	TraC	120	9.95	13,862.6		Membrane (2)
31	19745–20404	TraD	219	5.46	24,877.5		Soluble
32	20416–22434	TraE	672	5.50	77,233.8	COG1419	Soluble
33	22427–23845	TraF	472	9.10	53,277.4		Membrane (1)
34	23846–25003	Conserved hypothetical protein	385	6.66	41,739.7	COG0791	Membrane (1)
35	25017–25634	Conserved hypothetical protein	205	9.78	23,256.2		Membrane (1)
36	25621–25989	Conserved hypothetical protein	122	9.74	14,199.4	COG0526	Membrane (1)
37	25990–26460	TraJ	156	8.94	17,613.5		Membrane (2)
38	26462–28018	TraK	518	5.38	58,268.6	COG3505	Soluble
39	28033–28440	Conserved hypothetical protein	135	9.71	14,964.2		Membrane (1)
40	28442–29281	TraL	279	8.58	31,155.0		Membrane (6)
41	29296–29700	Hypothetical protein	134	9.27	16,000.8		Membrane (2)
42	29715–31853	TraI (topoisomerase)	712	9.30	80,062.9	COG0550	Soluble
43	31976–32191	Conserved hypothetical protein	71	4.14	7,826.8		Soluble
44	32195–33319	LtrC	374	7.79	42,313.5		Soluble

[a] Deduced by using the SOSUI web service (http://bp.nuap.nagoya-u.ac.jp/sosui/). Numbers of predicted transmembrane segments are shown in parentheses.

bility of plasmids (24). Almost identical resolvase genes were found in pSF118-44, *L. brevis* ATCC 367 plasmid 1, pCD01, and *L. casei* ATCC 334 plasmid 1. This may suggest a contribution of this type of resolvase to stable maintenance of pLgLA39 and these plasmids. While ORF17 is 95% identical to ORF12, it is 5′ truncated and likely inactive.

tra genes. The products of pLgLA39 ORF26 to ORF44 are similar (98.7% to 42.7% identical) to the *tra* gene products of *L. plantarum* pWCFS103 (46). These ORFs, except for ORF41, also have homologs in *L. lactis* subsp. *lactis* pMRC01 (83.2% to 37.2% identical) (9). Conjugation of pWCFS103 and pMRC01 has been reported (9, 46). The functions of the *tra* gene products, except for those of TraA (nicking enzyme) and TraI (topoisomerase), are not clear. A putative *oriT* sequence similar to those proposed for pMRC01 (9) and pWCFS103 (46) was found upstream of pLgLA39 *traA* (nt 15837 to 15861) (Fig. 2). Homologs of pLgLA39 ORF27, ORF28, ORF29, ORF30, ORF35, ORF37, ORF39, ORF41, and ORF43 are specific to these plasmids and an unnamed plasmid(s) of *L. paracasei* (8).

Other genes. ORF15 and ORF16 of pLgLA39 are almost identical to the genes for a Qac family multidrug resistance transporter and a TetR/AcrR-type transcriptional regulator, respectively, of *L. paracasei* pCD01 (8). ORF13 and ORF14 of pLgLA39 putatively encode a single polypeptide transposase. The putative insertion sequence (IS) containing ORF13 and ORF14 (nt 6580 to 7440 of pLgLA39) is 99.3% identical to IS*Lpl3* from *L. plantarum* WCSF1, which belongs to the IS*427* group of the IS*5* family (23). ISs nearly identical to the putative ISs of pLgLA39 and IS*Lpl3* are also found in plasmids (pRH45II and ATCC 367 plasmid 2) and the chromosome of *L. brevis* (ATCC 367). These may suggest transposing activity of this IS group.

Conjugative transfer of pLgLA39 from LA39 to *L. gasseri* JCM 1131[T]. pLgLA39 carries a set of *tra* genes, as described above. The conjugative ability of pLgLA39 was investigated. We succeeded in conjugative transfer of pLgLA39 from LA39 to *L. gasseri* JCM 1131[T] by filter mating. The transconjugant revealed elevated resistance to gassericin A and production of antibacterial activity.

JCM 1131[T] transformed with pSYE2 and thus resistant to Em was used as the recipient. The donor (LA39) and the recipient were filter mated, and transconjugants were selected on plates containing Em and gassericin A. JCM 1131[T] is sensitive to gassericin A (20), and the MIC for JCM 1131[T] of the 20×-concentrated culture supernatant of LA39 was 0.62% to 0.31% (vol/vol). To select transconjugants, 5% (vol/vol) gassericin A concentrate (approximately 10-fold the MIC) was used. The resulting clones, resistant to both Em and gassericin A, were further investigated. The clones grew in the presence of 20% (vol/vol) gassericin A concentrate. This level of resistance was >32-fold higher than that of JCM 1131[T] and comparable to that of the donor strain LA39. The clones harbored two plasmids: one sized similarly to pSYE2 and the other sized similarly to pLgLA39 (Fig. 1A, lane 3). Randomly amplified polymorphic DNA PCR of the clones gave a band pattern similar to that of JCM 1131[T] but distinct from that of LA39 (data not shown). These results indicated that the Em- and gassericin A-resistant clones were conjugants. We designated the conjugant strain JCM 1131[T](pLgLA39/pSYE2). The conjugant expressed significant antimicrobial activity (40 AU/ml), while the recipient strain JCM 1131[T] revealed no bacteriocin activity. This indicates that the *gaa* genes in pLgLA39 conferred to the conjugant cells productivity of gassericin A as well as immunity to gassericin A. Frequencies of conjugation were between 2.5×10^{-8} and 1.4×10^{-5} per donor cell and between 2.8×10^{-6} and 2.3×10^{-3} per recipient cell. As the donor/recipient ratio increased, the conjugation frequency per donor decreased while that per recipient increased. Frequencies per recipient cell were much higher than those per donor cell. This was due to a drastic reduction in CFU of the recipient cells during filter mating (data not shown), probably because gassericin A produced by the donor strain LA39 killed the recipient cells sensitive to gassericin A.

***L. reuteri* LA6 harbors a conjugal plasmid indistinguishable from pLgLA39.** Reutericin 6 is a circular bacteriocin with an amino acid sequence identical to that of gassericin A (17). *L. reuteri* LA6, a producer of reutericin 6, was proved to harbor a plasmid, designated pLrLA6 (Fig. 3A). pLrLA6 proved to be quite similar to pLgLA39 in several aspects. Conjugation of
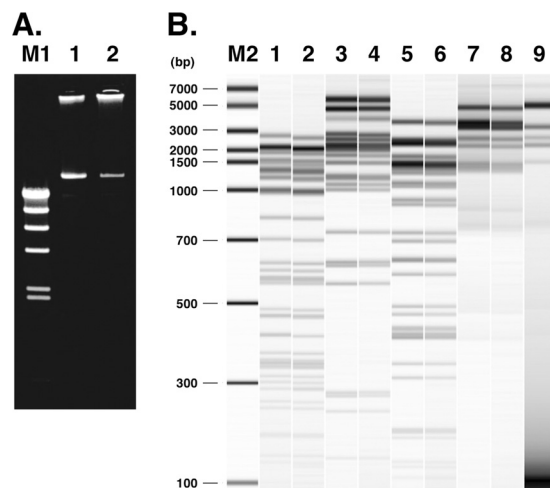

FIG. 3. Comparison of pLgLA39 and pLrLA6. (A) Agarose gel electrophoresis. Lanes: M1, λ HindIII digest; 1, pLgLA39 prepared from *L. gasseri* LA39; 2, pLrLA6 prepared from *L. reuteri* LA6. (B) Electrophoresis of restriction enzyme digests of pLgLA39 and pLrLA6 prepared from JCM 1131[T] transconjugants. Lanes: M2, DNA size marker (DNA7500 ladder; Agilent Technologies); 1 and 2, pLgLA39 and pLrLA6 digested with DraI; 3 and 4, digests with EcoT14I; 5 and 6, digests with HincII; 7 and 8, digests with HindIII; 9, HindIII digest of pLgLA39 prepared from LA39.

pLrLA6 from *L. reuteri* LA6 to *L. gasseri* JCM 1131[T] was successful by the method used for conjugation of pLgLA39 from *L. gasseri* LA39. In a conjugation experiment, conjugal frequencies were 4.8×10^{-8} per donor cell and 4.1×10^{-7} per recipient cell. pLrLA6 and pLgLA39 were prepared from the respective transconjugants, from which pSYE2 was cured, and were digested with the restriction enzymes DraI, EcoT14I, HincII, and HindIII. All of the digests exhibited similar band patterns for the two plasmids (Fig. 3B, lanes 1 to 8). In addition, PCRs done on pLrLA6 with the pLgLA39 sequence-based primer sets, which together cover the entire pLgLA39 sequence, gave amplicons of identical lengths to the corresponding amplicons from pLgLA39 (data not shown). In those aspects, pLrLA6 was indistinguishable from pLgLA39, and the two plasmids are probably almost identical to each other. Two regions of the plasmids were compared at the nucleotide sequence level. pLrLA6 carried a locus corresponding to the *gaa* genes of pLgLA39. The locus of pLrLA6 thought to be responsible for reutericin 6 production was designated *reu*. The nucleotide sequence of the *reu* region was determined to be 100% identical to that of the *gaa* region. Thus, not only the structural gene *reuA*, encoding the reutericin 6 precursor, but also the other six *reu* genes were completely identical to the corresponding *gaa* genes. *repA* and its upstream noncoding region of pLrLA6 were also identical to those of pLgLA39, except for a 1-nt insertion (T) in pLrLA6 (at nt 12555 of the pLgLA39 sequence).

To compare the restriction enzyme digests of pLrLA6 and pLgLA39 (Fig. 3B), we used plasmids prepared from JCM 1131[T], not from their original hosts. This eliminated possible unexpected effects by site-specific DNA modifications in the host cells (LA39 and LA6). Indeed, we observed site-specific modifications when HindIII was used. pLrLA6 prepared from
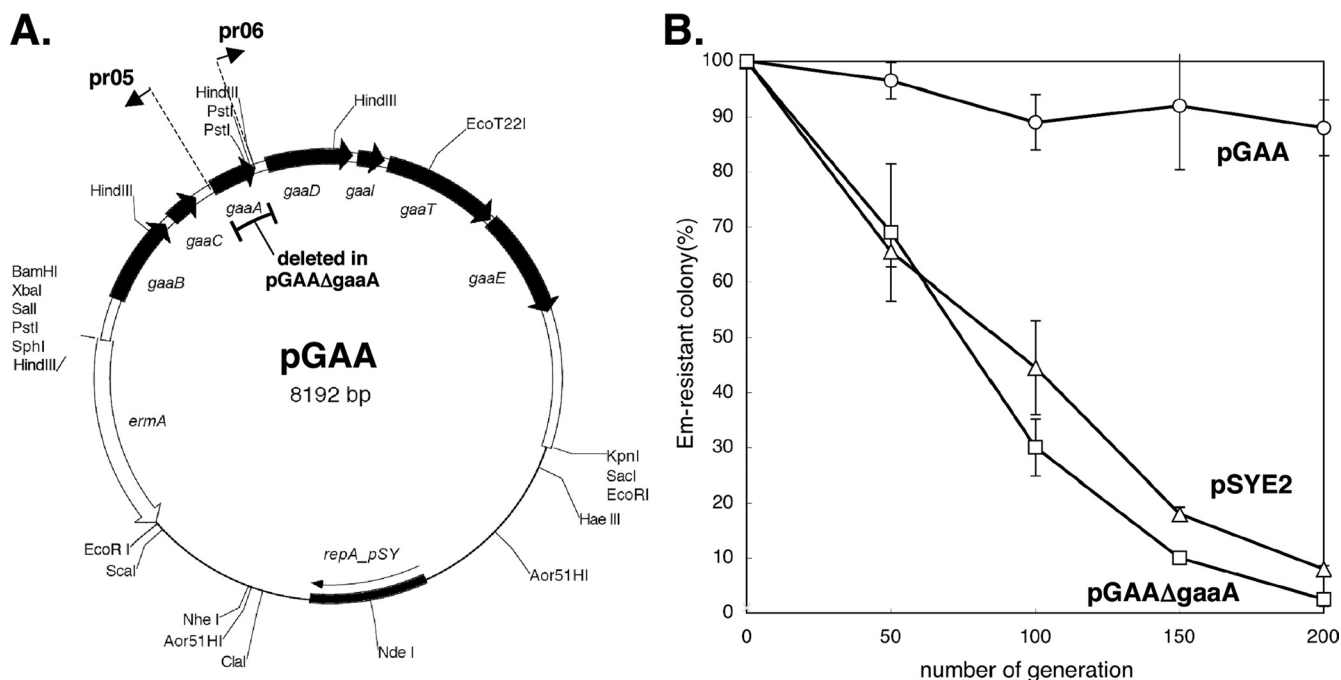
FIG. 4. Effect of gassericin A production on apparent plasmid segregational stability. (A) *gaa* expression plasmid pGAA and *gaaA*-deletant plasmid pGAAΔgaaA. Primers used in construction of pGAAΔgaaA are shown. The double-lined region is from pLgLA39. *ermA* indicates the 1,111-bp HhaI fragment of pIL253 (GenBank accession number AF041239) containing the Em resistance (adenine methylase) gene. *repA*_pSY indicates the replication initiator protein gene of pSY1 (GenBank accession number E05086). (B) Apparent segregational stabilities of plasmids in *L. gasseri* JCM 1131[T]. Symbols: circles, pGAA; squares, pGAAΔgaaA; triangles, pSYE2.

L. reuteri LA6 was not digested at all with HindIII, while it was sensitive to AluI (data not shown). This suggests the presence of M.HindIII-like N6A-methylase activity in LA6, and actually, from LA6 we cloned restriction and modification genes homologous to those of the HindIII system (unpublished results). When pLgLA39 prepared from *L. gasseri* LA39 was digested with HindIII, some specific HindIII sites were resistant to digestion (Fig. 3B, lane 9). In preliminary experiments, we determined that the protection occurred at HindIII sites preceded by G (5′-GAAGCTT-3′).

Cloning and expression of the *gaa* genes in JCM 1131[T] and enhancement of plasmid segregational stability by production of gassericin A. The seven *gaa* genes, *gaaBCADITE*, were cloned from pLgLA39 into pSYE2 to construct pGAA (Fig. 4A). *L. gasseri* JCM 1131[T] cells transformed with pGAA exhibited significant antibacterial activity (40 AU/ml). This indicates that the cloned *gaa* genes were active and produced gassericin A in the JCM 1131[T] background. Next, *gaaA* was removed from pGAA by inverse PCR to construct pGAAΔgaaA (Fig. 4A). JCM 1131[T] harboring pGAAΔgaaA produced no antibacterial activity, as expected. Both JCM 1131[T](pGAA) and JCM 1131[T](pGAAΔgaaA) were resistant to gassericin A, as they grew in the presence of 20% (vol/vol) gassericin A concentrate.

The apparent segregational stabilities of plasmids pGAA, pGAAΔgaaA, and pSYE2 in JCM 1131[T] were examined (Fig. 4B). pGAA was significantly (at a significance level of <0.05) more stable than pGAAΔgaaA and pSYE2 after 100 (P values of 0.0087 and 0.039, respectively) and 200 (P values of 0.017 and 0.025, respectively) generations. Copy numbers of the

three plasmids in JCM 1131[T] were determined by qPCR and were 22.8 ± 7.4 for pGAA, 27.0 ± 4.1 for pGAAΔgaaA, and 47.3 ± 16.4 for pSYE2. It should be noted that pGAA was more stable than pSYE2, which exists at approximately twice the number of copies of pGAA. The observed enhancement of the apparent segregational stability of pGAA by the *gaa* locus is probably a result of postsegregational host killing (PSK), as discussed below.

Functionality of pLgLA39-type *pemIK* as a plasmid-stabilizing mechanism. pLgLA39 ORF02 and ORF03 are *pemIK* homologs. The functionality of the pLgLA39-type *pemIK* system as a plasmid-stabilizing mechanism was confirmed experimentally. We had found *pemIK* in another *L. gasseri* plasmid, of which the translated amino acid sequences were 100% identical to those of PemIK of pLgLA39 (unpublished result). *pemIK* and *pemI* were PCR amplified from the plasmid and cloned into pSYE2+T to obtain pPemIK and pPemI, respectively (Fig. 5A). The cloned regions corresponded to nt 273 to 1263 and nt 273 to 718 of pLgLA39 (Fig. 2), respectively, and both contained a presumptive native promoter. The apparent segregational stabilities of pPemIK, pPemI, and pSYE2+T in *L. gasseri* JCM 1131[T] were compared (Fig. 5B). pPemIK was perfectly stable during the experiment and thus remarkably more stable than pPemI and pSYE2+T. Copy numbers determined for the three plasmids in JCM 1131[T] were nearly the same, as follows: 18.9 ± 4.3 for pPemIK, 16.5 ± 5.8 for pPemI, and 19.5 ± 1.2 for pSYE2+T. Therefore, the differences in apparent segregational stabilities were not due to differences in the quantities of the plasmids. These results indicate that the
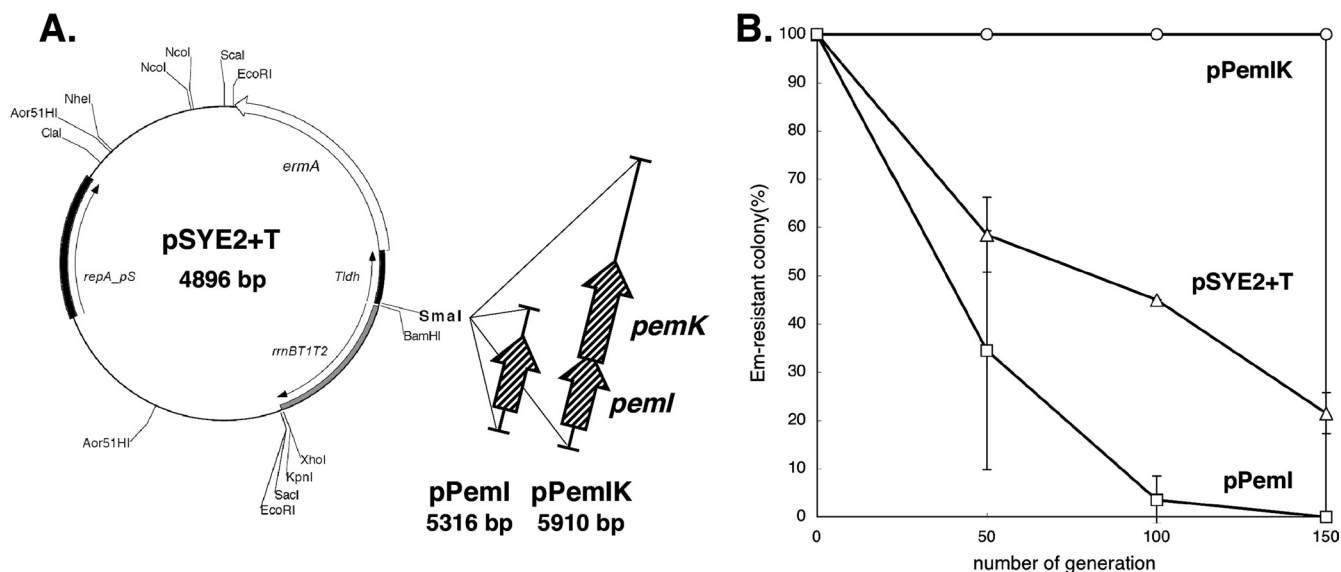
FIG. 5. Apparent plasmid segregational stabilization by pLgLA39-type *pemIK*. (A) pSYE2+T, pPemIK, and pPemI. See the text for details about construction of the plasmids. *Tldh* indicates the *S. thermophilus ldh* terminator. (B) Apparent segregational stabilities of plasmids in *L. gasseri* JCM 1131[T]. Symbols: circles, pPemIK; squares, pPemI; triangles, pSYE2+T.

pLgLA39-type *pemIK* system is functional as a plasmid-stabilizing mechanism.

Distribution of pLgLA39-related plasmids in *L. gasseri* strains. Of 27 *L. gasseri* strains examined, 17 strains exhibited possession of a plasmid(s). These plasmids varied in size and in restriction enzyme digestion patterns (data not shown). However, PCR using primers pr10 and pr11 and following DNA sequencing revealed that all of these plasmids carried a *repA* gene highly similar to pLgLA39 *repA*. This indicates a wide distribution in *L. gasseri* strains of plasmids belonging to the same replication type as pLgLA39. The amino acid sequences of RepAs of pLgLA39, the plasmids from eight independent *L. gasseri* strains, pSF118-44 from *L. salivarius* UCC 118, and plasmid 1 from *L. brevis* ATCC 367 were aligned (shown as a phylogram in Fig. 6A). The existence of the long nucleotide repeats and the short inverted repeat in the repeat unit was conserved in the *repA* genes from the eight *L. gasseri* plasmids (Fig. 6B). None of the plasmids from the 17 *L. gasseri* strains gave amplicons by PCR using primers pr01 and pr02 (data not shown). This indicates that these plasmids do not carry *gaaA*.

DISCUSSION

The gassericin A-producing strain *L. gasseri* LA39 was shown to harbor the 33,333-bp plasmid pLgLA39. This is the first report of the entire nucleotide sequence and detailed genetic analysis of an *L. gasseri* plasmid. Plasmids related to pLgLA39 are distributed in several *L. gasseri* strains as well as in other *Lactobacillus* and *Lactococcus* species. The seven *gaa* genes responsible for the production of gassericin A and immunity to gassericin A are located on pLgLA39, as opposed to the earlier localization of *gaaA* on the chromosome (19). pLgLA39 carries *tra* genes and was shown to conjugally transfer to *L. gasseri* JCM 1131[T]. The conjugant revealed productivity of gassericin A as well as resistance to gassericin A. Functional cloning of *gaa* in a small plasmid was achieved.

pGAA carrying *gaa* and expressing gassericin A was stably maintained. Apparent segregational stabilization of a plasmid by the pLgLA39-type *pemIK* system was also confirmed. Thus, pLgLA39 carries at least two independent stabilization mechanisms, the *gaa* and *pem* systems.

Reutericin 6 from *L. reuteri* LA6 is a circular bacteriocin whose amino acid sequence is identical to that of gassericin A (17). *L. reuteri* LA6 was shown to harbor a plasmid, pLrLA6, almost identical to pLgLA39. Furthermore, pLrLA6 contains seven genes that are 100% identical to the *gaa* genes of pLgLA39. *L. gasseri* LA39 and *L. reuteri* LA6 were isolated from feces of the same human infant within a 2-month interval (17, 19). These observations strongly suggest that pLgLA39 (pLrLA6) had naturally transferred between *L. gasseri* LA39 and *L. reuteri* LA6. Since *reu* and *gaa* are 100% identical to each other, the difference in the D-alanine contents of reutericin 6 and gassericin A (17) may be dependent on an as yet unidentified host factor(s).

The seven *gaa* genes were successfully cloned into plasmid vector pSYE2 to construct pGAA. The pGAA transformant of *L. gasseri* JCM 1131[T] produced significant bacteriocin activity. In contrast, JCM 1131[T] transformed with pGAAΔgaaA (a *gaaA*-depleted derivative of pGAA) did not produce bacteriocin activity. These results indicate that the bacteriocin produced by the pGAA transformant should be gassericin A. However, since gassericin A expressed in the JCM 1131[T] background was not chemically analyzed, it remains unclear whether it had the same characteristics, such as circularity, specific activity, and secondary and tertiary structures, as the authentic gassericin A produced by LA39. Analyses to confirm these points are in progress. To date, detailed molecular mechanisms for production of the class IV circular bacteriocins remain unclear. The production process includes maturation (digestion of precursor molecule), secretion, and circularization (covalent binding of N- and C-terminal ends). pGAA (8.2
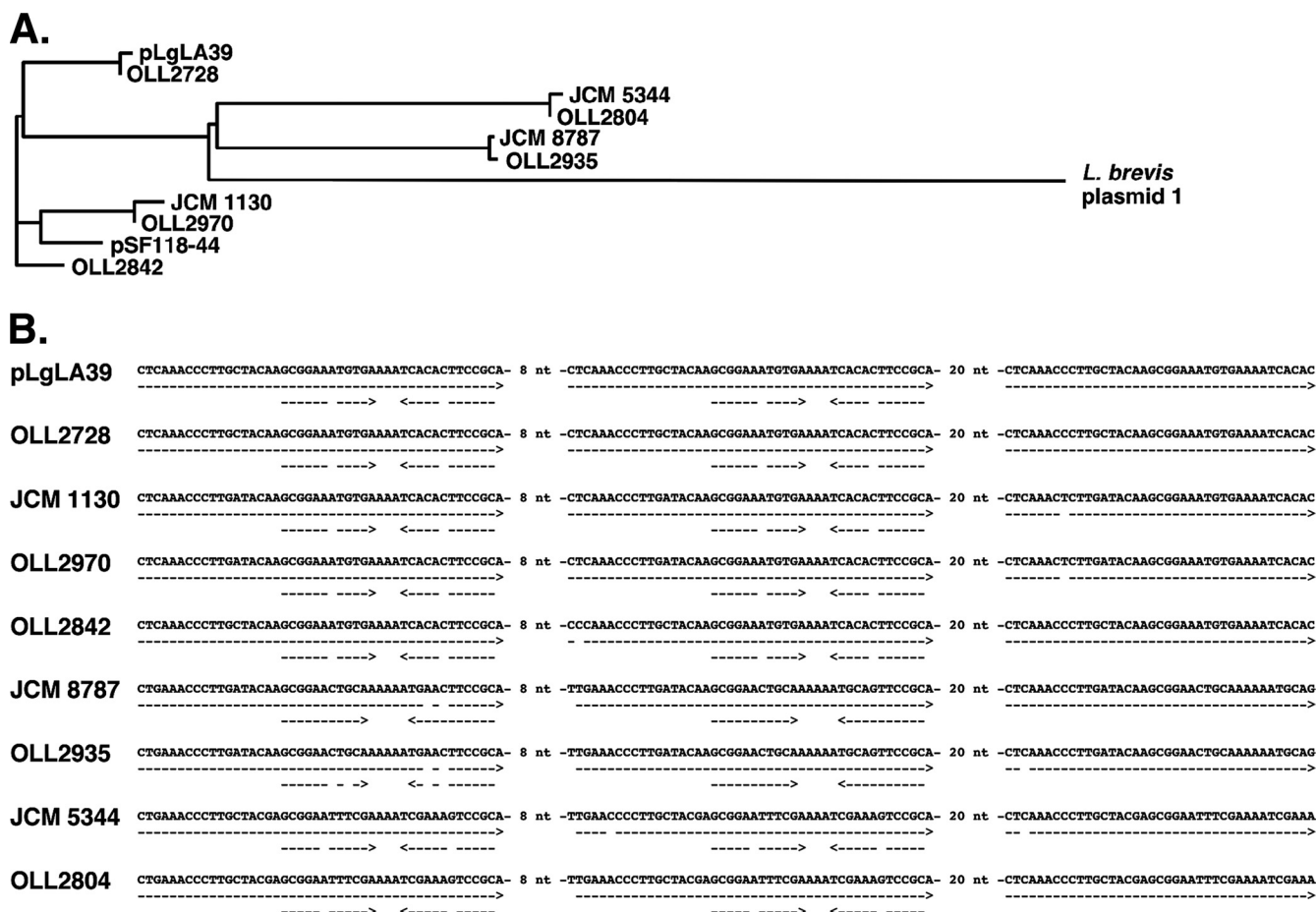
FIG. 6. pLgLA39-related plasmids in *L. gasseri* strains. (A) Phylogram based on amino acid sequence alignment of RepAs. (B) Comparison of repeat regions in *repA* genes of *L. gasseri* plasmids. Direct repeats and inverted repeats are indicated by dashed arrows. Positions of mismatches lack a dash.

kb) is small enough to be amplified by one PCR. As we demonstrated in the construction of pGAAΔgaaA, the inverse PCR technique can be applied to pGAA to inactivate a specific *gaa* gene(s) or to introduce a specific nucleotide alteration(s) into the *gaa* gene(s). This could facilitate functional analyses of the *gaa* genes to clarify molecular mechanisms underlying production, especially intramolecular circularization, of gassericin A, as well as immunity to the bacteriocin.

TA modules and restriction-modification systems are thought to be "selfish" genes since they cause PSK upon their loss from host cells (12). Both systems are often carried by bacterial plasmids and are thought to enhance the apparent segregational stability of the plasmids. We demonstrated that pGAA expressing gassericin A was more stably maintained in JCM 1131[T] than pGAAΔgaaA and even than the vector pSYE2 (Fig. 4B). This apparent stabilization can be explained by PSK; JCM 1131[T] cells having lost pGAA, and simultaneously immunity to gassericin A, are killed by gassericin A produced by neighbor cells retaining pGAA. In this context, bacteriocin genes should also be regarded as selfish. Three general features of bacteriocins are in accordance with this proposition. First, bacteriocins are usually active against bacteria closely related to the producers of the bacteriocins. This

could be plausible if the primary purpose of bacteriocins was to kill the cells that had once borne but then lost the bacteriocin genes. Second, so far as we know, no immunity mechanisms have been reported to inactivate cognate bacteriocins outside the producing cells by specifically degrading or capturing them. This may be because these mechanisms would help the sensitive cells to survive. Finally, bacteriocins are generally very stable. Above all, the class IV circular bacteriocins have no free ends and thus are even resistant to attacks by exopeptidases (6). Although the stability of immunity proteins has seldom been elucidated, they are likely less stable than cognate bacteriocins. Analogously, antitoxin proteins of TA modules are generally more fragile than toxin proteins, and the difference in the stabilities of the toxin and antitoxin is a prerequisite for PSK by TA systems.

The pLgLA39-type *pemIK* genes were also shown to be functional as a plasmid-stabilizing mechanism. *pem* in the R100 plasmid of *E. coli* is a well-studied TA system; PemK (toxin) is a site-specific RNase preferentially degrading mRNA at 5′-UA(C/A/U)-3′ sites, and the less stable PemI protein (antitoxin) blocks the nuclease activity of PemK (49). TA systems similar to the *E. coli pem* system are widely distributed among bacterial plasmids and chromosomes. We showed that

the pLgLA39-type *pemIK* system cloned into the rolling-circle-replicating plasmid pSYE2+T drastically increased the apparent segregational stability of the resulting plasmid, pPemIK (Fig. 5A). Since the basic mechanism of TA systems is independent of the type of replicon, *pemIK* of pLgLA39 likely enhances the apparent segregational stability of pLgLA39. *pemIK* of pLgLA39 can be used as a stabilizer for unstable plasmids or for plasmids with antibiotic resistance markers in cases where the usage of antibiotics is undesirable. Recently, another *pemIK* homolog in pSF118-20 from *L. salivarius* UCC 118 was reported to function as a TA system (10). The PemK proteins of pLgLA39 and pSF118-20 are 41.6% identical to each other over the entire amino acid sequence, while the PemI proteins of the two plasmids show lower and partial similarity (33.3% identical in 63 aa over 81 aa). PemK and PemI of pLgLA39 revealed even lower amino acid sequence similarities to the proteins encoded by *E. coli* R100. It should be elucidated further whether PemK and PemI of pLgLA39 work via a similar mechanism to the *E. coli pem* system and other heterologous *pem* systems, such as that in pSF118-20.

We showed that several *L. gasseri* strains harbor a plasmid(s) with a *repA* gene highly homologous to *repA* of pLgLA39. The *gaa* genes, however, are not carried by these plasmids. We determined the draft genome sequence of *L. gasseri* strain OLL2716 (39) and found seven genes on the chromosome with significant similarities to the *gaa* genes (translated amino acid sequence identities of 32% to 57% [unpublished results]). The chromosomal *gaa*-like genes were found neither in LA39 nor in the genome sequence of *L. gasseri* ATCC 33323 (3). Although it remains to be elucidated whether the *gaa*-like genes are expressed in OLL2716, the bacteriocin might be a new member of the gassericin A group of circular bacteriocins.

pLgLA39 is the first entirely sequenced plasmid from *L. gasseri*. For genetic manipulation of the species, mainly plasmid vectors of lactococcal origin have been used (27). Since pLgLA39 is supposed to replicate via the θ mechanism, high segregational and structural stability is expected. The wide distribution of pLgLA39-related plasmids in *Lactobacillus* and *Lactococcus* species suggests that vectors based on pLgLA39 *repA* could be applicable to many lactic acid bacteria. We successfully constructed a plasmid consisting of exclusively pLgLA39 *repA* (nt 12604 to 13741) and *ermA*, and the plasmid was maintained in *L. gasseri* JCM 1131^T and also in *L. lactis* IL1403 (unpublished results). Furthermore, employment of a complementary selection marker gene originating in *L. gasseri* would enable a self-cloning vector for the species and could be used in food-grade genetic manipulation of probiotic *L. gasseri* strains.

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