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Homologous Expression and Characterization of Gassericin T and Gassericin S, a Novel Class IIb Bacteriocin Produced by *Lactobacillus gasseri* LA327

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ABSTRACT *Lactobacillus gasseri* LA327, isolated from the large intestine tissue in humans, is a bacteriocinogenic strain with two kinds of class IIb bacteriocin structural genes, i.e., those for gassericin T (GT) and acidocin LF221A (Acd LF221A). In this study, DNA sequencing of the genes for GT and Acd LF221A from *L. gasseri* LA327 revealed that the amino acid sequences for GT corresponded with those for GT genes, except for GatK (histidine kinase). However, Acd LF221A genes had analogues which differed in at least one amino acid residue, to encode a class IIb bacteriocin designated gassericin S (GS). The LA327 strain retained antimicrobial activity after the deletion of the GT structural genes (*gatAX*); however, both GS and GT activities were lost by deletion of the putative ABC transporter gene (*gatT*). This indicates that the LA327 strain produces GS and GT and that GS secretion is performed via GT genes with the inclusion of *gatT*. Homologous expression using deletion mutants of GS and GT, each containing a single peptide, elucidated that GS (GasAX) and GT (GatAX) showed synergistic activity as class IIb bacteriocins and that no synergistic activity was observed between GS and GT peptides. The molecular mass of GS was estimated to be theoretical ca. 5,400 Da by *in situ* activity assay after SDS-PAGE, clarifying that GS was actually expressed as an active class IIb bacteriocin. Furthermore, the stability of expressed GS to pH, heat, and protease was determined.

IMPORTANCE Bacteriocins are regarded as potential alternatives for antibiotics in the absence of highly resistant bacteria. In particular, two-peptide (class IIb) bacteriocins exhibit the maximum activity through the synergy of two components, and their antimicrobial spectra are known to be relatively wide. However, there are few reports of synergistic activity of class IIb bacteriocins determined by isolation and purification of individual peptides. Our results clarified the interaction of each class IIb component peptide for GT and GS via the construction of homologous mutants, which were not dependent on the purification. These data may contribute to understanding the mechanisms of action by which class IIb bacteriocins exhibit wide antibacterial spectra.

KEYWORDS *Lactobacillus gasseri*, antimicrobial peptide, bacteriocin, homologous expression, lactic acid bacteria, multiple transporter, synergistic effect

Lactic acid bacteria (LAB) have a long history of consumption by humans and inhabit various ecosystems, including gastrointestinal (GI) tracts of humans and animals and other environments (for instance, vegetables, milks, and meats). In addition, lactobacilli are recognized as important members of the beneficial GI microbiota in

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humans and animals, along with bifidobacteria (1). In particular, *Lactobacillus gasseri* is one of the predominant species of lactobacilli in the human small intestine, and it has been isolated not only from the GI tract and feces but also from the oral and the vaginal cavities and mammary areola. It is considered a promising candidate for probiotics (2–5).

Many researchers have reported the beneficial effects of *L. gasseri* on hosts, including immunoregulation, the alleviation of allergic symptoms, prevention of bacterial and viral infections, the antitumor effect, and the inhibition of lipid absorption (6–11). Indeed, at least three yogurt brands and one supplement containing *L. gasseri* strains are commercially sold in Japan to provide the benefits mentioned above to consumers. It has been known that LAB may produce various antimicrobial agents, such as lactic acids, diacetyl, hydrogen peroxide, and bacteriocins, to survive in competitive microbial niches (12–14).

Bacteriocins are defined as ribosomally synthesized antimicrobial peptides and proteins from microorganisms (15). LAB bacteriocins especially are regarded as a potential alternative to antibiotics due to several advantageous properties, such as their potency (as determined *in vitro* and *in vivo*), variety of antimicrobial spectra (both narrow and broad), low toxicity, and ease of the bioengineering (16). Presently, bacteriocins from LAB are classified into three major classes: ribosomally produced and posttranslationally modified peptides (RiPPs) (less than 10 kDa, class I), unmodified bacteriocins less than 10 kDa (class II), and unmodified bacteriocins greater than 10 kDa (class III). Furthermore, class I is subclassified into six subclasses: class Ia (lanthipeptides), class Ib (head-to-tail cyclized peptides), class Ic (sactibiotics), class Id [linear azol(in)e-containing peptides], class Ie (glycocins), and class If (lasso peptides). Additionally, class II is subclassified into four subclasses: class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), class IIc (leaderless bacteriocins), and class IId (non-pediocin-like, single-peptide bacteriocins) (17).

Previously, we reported that gassericin A (GA), a head-to-tail cyclized bacteriocin (class IId) from *L. gasseri* LA39 (18), and gassericin T (GT), a putative two-peptide bacteriocin (class IIb), consisted of GatA and GatX from *L. gasseri* SBT2055 and LA158 (19, 20). In addition, Bogovic-Matijašić et al. (21) reported that acidocin LF221A (Acd LF221A) and acidocin LF221B (Acd LF221B) were produced by *L. gasseri* LF221; the latter was an analogue of GatX (the second peptide of GT) containing alanine at position 50 instead of glycine in GatX, and the former was potentially a novel bacteriocin derived from *L. gasseri*. Recently, we isolated *L. gasseri* LA327 harboring the structural genes for GT (*gatAX*) and Acd LF221A (*acd 221*α*A*), detected by PCR from human large intestine tissue. In addition to GT, Acd LF221A, and Acd LF221B, gassericin K7A and gassericin K7B from *L. gasseri* K7 and gassericin E from *L. gasseri* EV1461 have been reported as class IIb bacteriocins produced by *L. gasseri* (22–24). The individual peptides of Acd LF221A (Acd 221A), Acd LF221B (Acd 221B), gassericin K7A (GasK7A_β), gassericin K7B (GasK7B_β), and gassericin E (GaeA) have antibacterial activity, and no increase of the activity was recognized by combination assay with Acd 221A and Acd 221B. However, complete purification of two peptides of class IIb bacteriocins produced by *L. gasseri* has never been achieved, and the synergistic activity produced by coexistence of each peptide, which should be an essential character for class IIb bacteriocin, has still not been demonstrated.

In this study, we focused on identification and characterization of two class IIb bacteriocins detected from *L. gasseri* LA327 and demonstration of the two-component action through homologous expression of these bacteriocins using genetically constructed mutant strains.

RESULTS

DNA sequencing and genetic analysis. The nucleotide sequencing of the region surrounding *gatAX* and *gasAX* (like that for acidocin LF221A and gassericin K7A) on the chromosomal DNA of *Lactobacillus gasseri* LA327 highlighted 6,935-bp and 1,143-bp DNA sequences harboring nine and three open reading frames (ORFs) (DDBJ accession no. LC389592 and LC389591), respectively. Homology searching of the predicted amino

TABLE 1 Deduced peptides and proteins encoded in *gat* operon of *Lactobacillus gasseri* LA327

ORF	Protein or peptide					
	Length (amino acids)	Mol wt	pI	Function	Homology search result[a] (identity [residual ratio, %])	Localization (no. of TMS)[b]
gatP	50	5,852	9.83	Inducer peptide precursor	Lactacin F inducer peptide precursor of *L. gasseri* K7 (50/50, 100)	Soluble (0)
gatK	435	50,444	5.2	Histidine kinase	Histidine kinase of *L. gasseri* K7 (430/435, 99)	Membrane (6)
gatR	265	30,280	5.36	Response regulator	Chemotaxis protein CheY of *L. gasseri* K7 (265/265, 100)	Soluble (0)
gatT	719	81,054	7.71	ABC transporter permease component	Peptide ABC transporter ATP-binding protein of *L. gasseri* K7 (718/719, 99)	Membrane (6)
gatC	197	21,938	10.13	Putative accessory protein	Putative gassericin K7 B accessory protein (196/197, 99)	Membrane (1)
gatZ	33	3,924	7.57	Putative uncharacterized protein	Unknown protein of *L. gasseri* LF221 (33/33, 100)	Soluble (0)
gatA	75	7,480	10.02	GatA precursor	Putative complement factor Acd 221b of *L. gasseri* LF221 (75/75, 100)	Membrane (1)
gatX	65	6,673	10.11	GatX precursor	Acidocin LF221B Acd 221B of *L. gasseri* LF221 (64/65, 98)	Soluble (0)
gatI	112	13,343	9.81	Immunity protein	Putative immunity protein Aci 221B of *L. gasseri* LF221 (112/112, 100)	Membrane (4)

[a]Homology searching was done with the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html).
[b]Deduced using the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/).

acid sequences for peptides and proteins encoded in each ORF revealed that the nine products for the *gat* operon matched those of *L. gasseri* LA158 (GenBank accession no. AB710328), with the exception of one amino acid residue in GatK (the amino acid residue at position 168 is valine in LA327 instead of alanine in LA158 [data not shown]), and the three ORFs for the *gas* operon were not detected in LA158. Furthermore, the *gas* and *gat* operons were similar to those of acidocin LF221A (Acd LF221A) and gassericin K7A and to those of acidocin LF221B (Acd LF221B) and gassericin K7B, respectively (Tables 1 and 2). However, the sequences of two class IIb bacteriocins for the *gas* and *gat* operons in *L. gasseri* LA327 were slightly different (in the range of 0 to 2 amino acid residues) from those for Acd LF221A and Acd LF221B (GenBank accession no. AY295874.1 and AY297947.1) (Fig. 1A and B), so the new putative bacteriocin (derived from the *gas* operon) was designated as gassericin S (GasA and GasX).

Verification of the GS production mechanism. GT activity (15,754 arbitrary units [AU]/ml) in the culture supernatant (cell-free supernatant [CFS]) of *L. gasseri* LA158 was completely lost by deletion of the GT structural genes (*gatAX*) from chromosomal DNA of LA158 (LA158 Δ*gatAX*), and the bacteriocin activity of *L. gasseri* LA327 was retained and increased (from 16 AU/ml to 64 AU/ml) after *gatAX* was eliminated (LA327 Δ*gatAX*). However, the bacteriocin activity in the CFS of LA327 ceased after elimination of the gene for the putative ABC transporter (*gatT*) from the chromosomal DNA of LA327 (LA327 Δ*gatT*), suggesting that gassericin S (GS) is a bacteriocin secreted through GatT.

Synergistic activity among the peptides of GS and GT. Bacteriocin activities (62 AU/ml and 123 AU/ml) were detected in the CFSs of coproducer strains for GasAX [*L. gasseri* LA158 Δ*gatAX*(pGS-AX) and LA158 Δ*gatAX*(pGS-AXI), respectively], and no activities were obtained in the CFSs of single-producer strains for GasA and GasX [*L. gasseri* LA158 Δ*gatAX*(pGS-AXΔX), LA158 Δ*gatAX*(pGS-AXIΔX), LA158 Δ*gatAX*(pGS-AXΔA), and LA158 Δ*gatAX*(pGS-AXIΔA)], even when concentrated 40-fold (Table 3). On

TABLE 2 Deduced peptides and proteins encoded in the *gas* operon of *Lactobacillus gasseri* LA327

ORF	Protein or peptide					
	Length (amino acids)	Mol wt	pI	Function	Homology search result[a] (identity [residual ratio, %])	Localization (no. of TMS)[b]
gasA	79	7,662	10.27	GasA precursor	Gassericin K7 A complementary factor (78/79, 99)	Membrane (1)[c]
gasX	69	7,287	10.00	GasX precursor	Acidocin LF221A Acd 221A (67/69, 97)	Membrane (1)[c]
gasI	90	10,789	7.42	Putative immunity protein	Gassericin K7 A immunity protein (90/90,100)	Membrane (2)

[a]Homology searching was done with the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html).
[b]Deduced using the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/).
[c]With putative mature peptide.

FIG 1 (A) Comparison of deduced amino acid sequences for gassericin S (GasA and GasX) and acidocin LF221A (Acd 221α and Acd 221A). Arrows, underlining, and bold indicate predicted cleavage sites, putative transmembrane domains, and amino acid residue differences between GS and Acd LF221A, respectively. (B) Comparison of deduced amino acid sequences for gassericin T (GatA and GatX) and acidocin LF221B (Acd 221β and Acd 221B). Arrows, underlining, and bold indicate predicted cleavage sites, putative transmembrane domains, and amino acid residue differences between GT and Acd LF221B, respectively.

the other hand, a mixture of equal amounts of GasA (GasAI) and GasX (GasXI) showed bacteriocin activity (Table 3).

Similarly, the assay for combination activity of component peptides of GT (GatA and GatX) in the CFSs of single-producer strains for GatA and GatX (*L. gasseri* LA158 Δ*gatX* and LA158 Δ*gatA*) clarified that GatA displayed activity (62 AU/ml) when alone, and remarkably high activity (1,969 AU/ml) was observed with a combination of GatA and GatX (Table 4). In the combination activity assay between each GS and GT component peptide, a remarkably high activity level was not observed (Table 5).

TABLE 3 Antibacterial activities of Gas peptides in alone and combination

CFS[a]	Bacteriocin activity[b] (AU/ml)
GasA	ND[c]
GasX	ND
GasAI	ND
GasXI	ND
GasAX	62
GasAXI	123
GasA + GasX (1:1)	62
GasA + GasXI (1:1)	31
GasAI + GasX (1:1)	62
GasAI + GasXI (1:1)	31

[a]GasA, cell-free supernatant (CFS) of *L. gasseri* LA158 Δ*gatAX*(pGS-AXΔX); GasX, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXΔA); GasAI, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXIΔX); GasXI, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXIΔA); GasAX, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AX); GasAXI, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXI).

[b]$n > 3$. Indicator, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2).

[c]ND, not detected. Approximately 40-fold-concentrated CFS was used.

TABLE 4 Combined activity of GatA and GatX

CFS[a]	Bacteriocin activity[b] (AU/ml)
GatA	62
GatX	ND[c]
GatA + GatX (1:1)	1,969

[a]GatA, CFS of *L. gasseri* LA158 Δ*gatX*; GatX, CFS of *L. gasseri* LA158 Δ*gatA*.
[b]$n > 3$. Indicator, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2).
[c]ND, not detected. Approximately 40-fold-concentrated CFS was used.

Synergistic activities of GS (GasA plus GasX) and GT (GatA plus GatX) were detected with isobolograms (Fig. 2 and 3), while no synergistic activity was obtained for the combination of GS (GasA plus GasX) plus GT (GatA plus GatX) (Fig. 4).

***In situ* activity assay for GS.** A clear zone derived from GS appeared in the *in situ* activity assay after SDS-PAGE, and the molecular mass of GS was found to be approximately 5,400 Da by using a standard curve constructed with the electrophoresis distance of each molecular marker (Fig. 5).

GS stability against pH, heat, and proteolysis. The stability of GS under various pH, thermal, and proteolytic conditions was investigated. The bacteriocin activity of GS in the CFS was stable in a wide pH range (pH 2, 4, 7, and 10) (data not shown), and 25% of GS activity was retained under the most severe heating condition (121°C, 15 min) used in the tests (Table 6). The GS activity decreased to 25% under incubation at 37°C but was maintained at 4°C (Table 6). Through examination of the proteolysis resistance of GA, GT, and GS, it was determined that there were no bacteriocins showing tolerance against proteinase K. GS completely lost activity upon treatment with all proteases used in this study, and GT had a slight tolerance only for pepsin. On the other hand, the bacteriocin activity of GA, a circular bactericidal peptide, was detected after treatment with all proteases used, with the exception of proteinase K (Table 7).

DISCUSSION

In this study, we determined the complete sequence of a gassericin S (GS) gene cluster constituted by three ORFs containing the structural genes (*gasAX*) and the predicted immunity gene (*gasI*). Although the genes encoding bacteriocin precursors, immunity peptides/proteins (frequently found near the structural gene [25]), and transporter proteins are required for bacteriocin synthesis (26), no genes related to regulation and transport of GS were recognized in the surrounding areas of the *gas* operon. Deletion of the gassericin T (GT) transporter gene (*gatT*) clarified that GatT works as a multiple transporter for GT and GS, like EnkT which is responsible for the secretion of three enterocins from *Enterococcus faecium* NKR-5-3 (27). The complete isolation and purification of class IIb bacteriocins from *Lactobacillus gasseri* were not achieved, and synergy between the component peptides has never been reported. In this study, we constructed recombinant strains expressing a single peptide of GS and GT and demonstrated the existence of synergistic activities for each gassericin (Fig. 2 and 3), indicating that GS and GT are class IIb bacteriocins.

Majhenič et al. (28) reported that acidocins LF221A and LF221B, which have two- and one-amino-acid variants of the second peptides (GasX and GatX) for GS and GT,

TABLE 5 Combined activity of GS and GT components

CFS[a]	Theoretical activity (AU/ml)	Actual activity[b] (AU/ml)
GasA + GatA	(ND[c] + 62)/2	31
GasA + GatX	(ND + ND)/2	ND
GasX + GatA	(ND + 62)/2	31
GasX + GatX	(ND + ND)/2	ND
GasA + GasX + GatA + GatX	(123 + 1,969)/2	985

[a]GasA, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXIΔX); GasX, CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXΔA); GatA, CFS of *L. gasseri* LA158 Δ*gatX*; GatX, CFS of *L. gasseri* LA158 Δ*gatA*. CFSs were used in equal amounts.
[b]$n > 3$. Indicator, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2).
[c]ND, not detected.

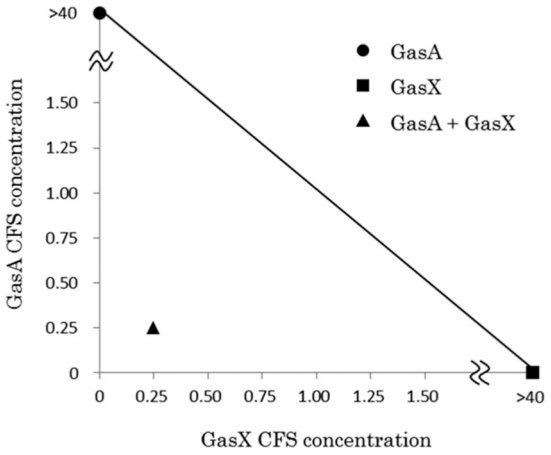

FIG 2 Isobologram assay of GS (GasA plus GasX). CFS, cell-free supernatant. Concentrations: 1, undiluted CFS; <1, diluted CFS; >1, concentrated CFS. All data for bacteriocin activities were measured with at least triplicates.

respectively, inhibited the growth of *Lactobacillus sakei* NCDO2714 when isolated. Although difference in the indicator strains used may be related to the different results, they are more likely due to the variation of the amino acid residues in acidocin LF221A and LF221B. Influences of single-amino-acid variation between the second peptides of enterocin C and enterocin 1017 on their antimicrobial spectra have been clarified (29). However, inhibition against *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T (JCM 1002^T) of the second peptide for gassericin K7B, 100% homologous to the GT second peptide, was reported (23), indicating that the structure of the gassericin K7B second peptide (active) may be different from that of the GT second peptide (inactive) in this study, owing to the test solutions and/or expression hosts used, in spite of the same indicator strain and amino acid sequences being used. Component peptides of the class IIb bacteriocin carnobacteriocin XY showed a high α-helix content in trifluoroethanol solution, while neither peptide showed remarkable secondary structure in aqueous conditions (30). Furthermore, our previous attempts to isolate and purify individual GT peptides from the CFS of *L. gasseri* LA158 failed because of polymerization of the peptides in 60% 2-propanol at the final step for high-pressure liquid chromatography (HPLC) purification (20). Therefore, the organic solvents used for bacteriocin extraction may be involved in the finding that individual activity of a second peptide

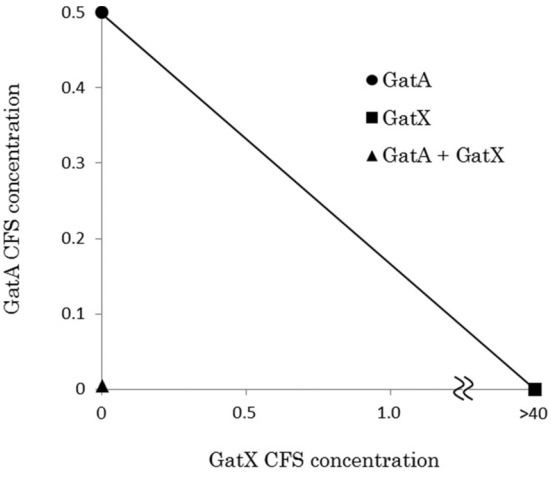

FIG 3 Isobologram assay of GT (GatA plus GatX). CFS, cell-free supernatant. Concentrations: 1, undiluted CFS; <1, diluted CFS; >1, concentrated CFS. All data for bacteriocin activities were measured with at least triplicates.

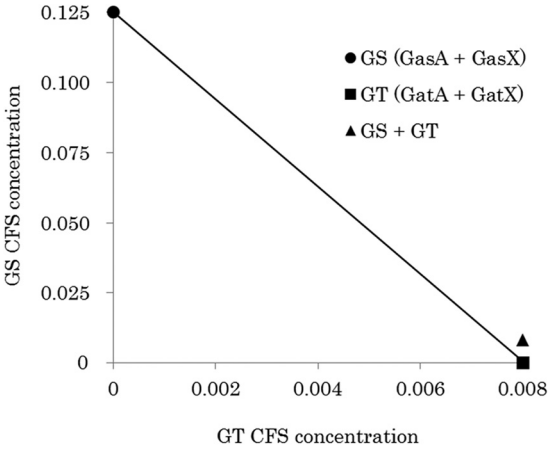

FIG 4 Isobologram assay of GS and GT. CFS, cell-free supernatant. Concentrations: 1, undiluted CFS; <1, diluted CFS; >1, concentrated CFS. All data for bacteriocin activities were measured with at least triplicates.

of each class IIb bacteriocin from *L. gasseri* has been reported. In contrast, the activity exhibited by only a first peptide of gassericin E (one-amino-acid variants of the GT first peptide, W22L) (24) supports that the GT first peptide has antibacterial activity alone. Moreover, in this study, a putative immunity protein, GasI, promoted the production of GS, while production of individual peptides for GS was not affected by *gasI* expression (Table 3). Ra et al. (31) reported that deletion of *nisI*, encoding the immunity protein of nisin A, led to a decrease in nisin production. These results indicate that the resistance of GasI may be unnecessary for production of each inactive single peptide for GS, while GasI supports active GS production.

It is not rare that multiple bacteriocins are produced by a single strain, but many questions about the advantages of this still remain. In this study, synergistic activity of a cross-combination of GT and GS was not shown (Fig. 4). Similarly, no synergistic activity was observed in a cross-combination of plantaricin EF and plantaricin JK (32), but high activity was obtained in the case of the hemilateral peptide of lactococcin G and the complementary peptide of enterocin 1017 and lactococcin Q (33–35). It seems

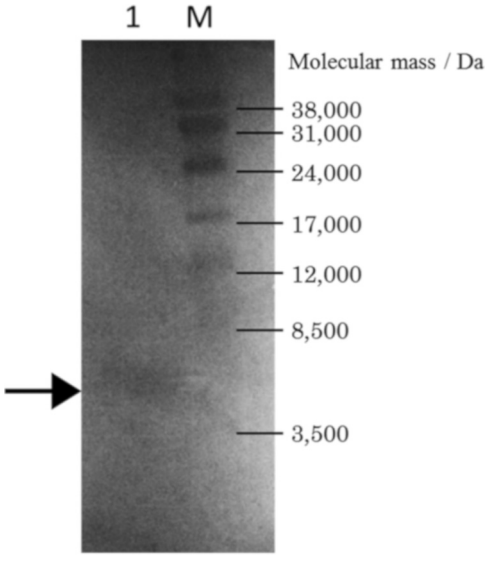

FIG 5 *In situ* activity assay of semipurified gassericin S. Indicator, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2). Lane M, molecular weight markers; lane1, semipurified gassericin S. The arrow indicates the clear zone of gassericin S.

TABLE 6 Heat stability of gassericin S[a]

Condition	Bacteriocin activity,[b] AU/ml (residual rate, %)
Untreated	123
121°C, 15 min	31 (25)
95°C, 5 min	62 (50)
70°C, 1 h	62 (50)
37°C, 1 mo	31 (25)
4°C, 1 mo	123 (100)

[a]Determined with the CFS of *L. gasseri* LA158 Δ*gatAX*(pGS-AXI).
[b]$n > 3$. Indicator, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2).

that presentation of cross-combination activity is dependent on amino acid sequence similarity, as demonstrated by the low similarity of GS and GT (ca. 26% as calculated by BLAST [https://blast.ncbi.nlm.nih.gov/Blast.cgi]) and the high similarity of lactococcin G and enterocin 1017 or lactococcin Q (ca. 88% and 57%, respectively).

After tests at various pHs and temperatures, the bacteriocin activity of GS produced by *L. gasseri* LA158 Δ*gatAX*(pGS-AXI) remained, even when the CFS was exposed to pH 2 to 10 (data not shown) and heated at 121°C for 15 min (Table 6). However, the activity decreased remarkably with longer incubation at 37°C, which differed from holding at 4°C (Table 6). In our previous study, the bacteriocin activity of GT produced by LA158 similarly decreased during incubation at 37°C, but the rate of decay of the activity was weakened after heat treatment of the CFS containing GT at 99°C for 10 min (data not shown), suggesting that the degradation of GT and GS activity may be caused by an extracellular protease(s) derived from LA158, used as the GS expression host.

In proteolysis sensitivity testing, the activities of GT and GS were greatly reduced and then completely disappeared with all proteases used in the test, and GA had a high protease tolerance, as expected from reports that peptides and proteins having cyclic structures are generally stable when exposed to heat, denaturant, and protease treatments (36). These characteristics would be available for application at various stages through the combination of GA and GT/GS. For instance, we reported that GA and GT prevented the growth of *Staphylococcus aureus* isolated from mastitis milk and breast organs (37, 38). In particular, GA is expected to sustain bacteriocin activity, due to its high stability, not only as a food preservative but also as a mastitis therapeutic agent, and GT and GS may be used as safe food preservatives that will be degraded in the human intestine after intake because of their high sensitivity to proteinases. Although most cleavage sites of pepsin (FYWML) overlap with those of alpha-chymotrypsin (FYW) and proteinase K (FYWMC), the effects of each protease treatment on the activities of GA, GT, and GS were different (Table 7; Fig. 6), indicating that the amounts of protease units and tertiary structures of gassericins, rather than cleavage sites, may be important for maintaining bacteriocin activities. The elucidation of the antibacterial spectra, mode of action, and immunity system for GS is still in progress.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are summarized in Table 8. The gassericin T (GT) producer *Lactobacillus gasseri* LA158 was isolated from the feces of a

TABLE 7 Proteolysis sensitivity of bacteriocins produced by *Lactobacillus gasseri*

Protease	Residual activity of bacteriocin, AU/ml (rate, %)[a]		
	GA	GT	GS
None	985	31,508	246
Pepsin	492 (50)	492 (1.6)	ND[b]
Trypsin	62 (6.3)	ND	ND
Alpha-chymotrypsin	246 (25)	ND	ND
Proteinase K	ND	ND	ND

[a]$n > 3$. Indicator, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2). GA, gassericin A (CFS of *L. gasseri* LA39); GT, gassericin T (CFS of *L. gasseri* LA158); GS, gassericin S (CFS of *L. gasseri* LA327).
[b]ND, not detected.

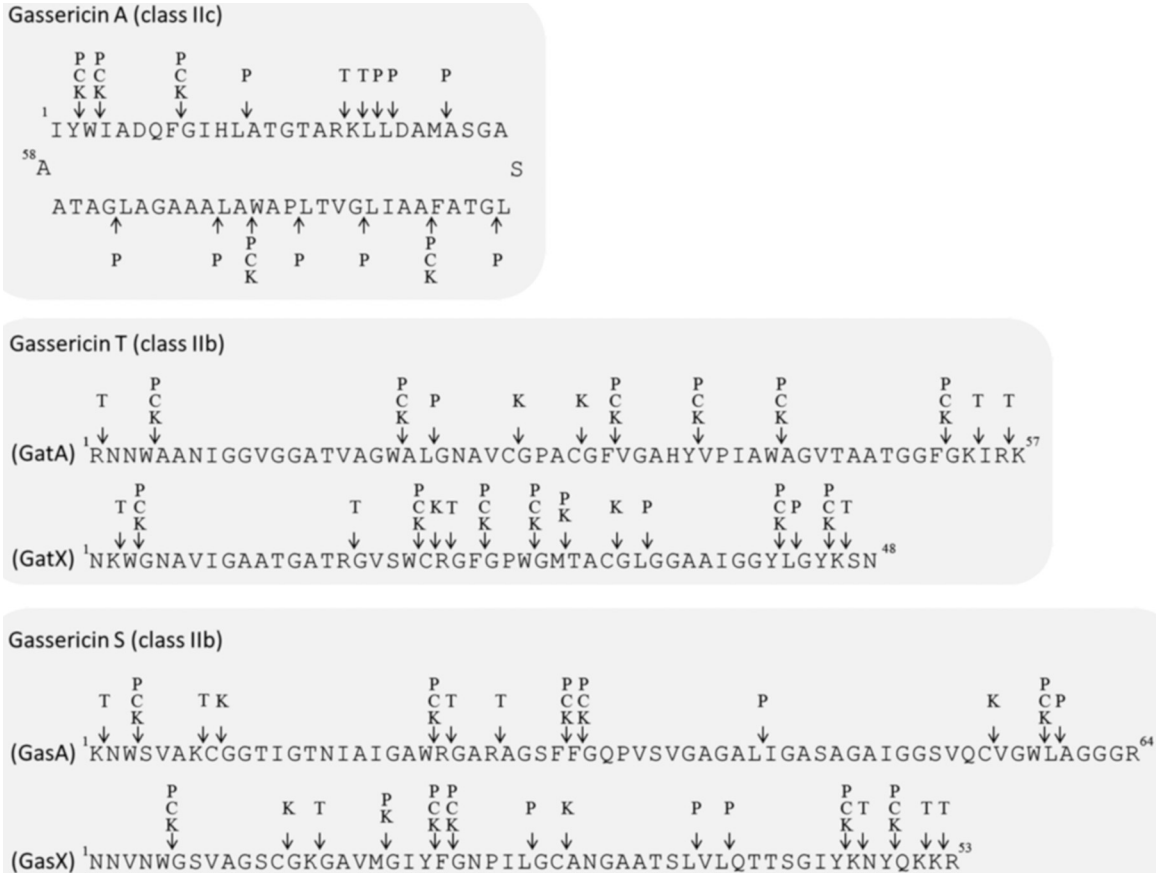

FIG 6 Predicted cleavage sites for each protease on gassericins A, T, and S. Arrows indicate the predicted cleavage sites for each protease (P, pepsin; C, alpha- chymotrypsin; K, proteinase K; T, trypsin).

six-month-old female human infant. The gassericin S (GS) and GT producer *L. gasseri* LA327 was isolated from the large intestine of an adult human. *L. gasseri* strains and the indicator strain, *Lactobacillus delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2), were cultivated in MRS broth (Becton Dickson, MD, USA) and cultured at 37°C. The intermediate-expression host strains *Lactococcus lactis* subsp. *cremoris* MG1363 and *Lactococcus lactis* subsp. *lactis* IL-1401 were cultivated in M17 medium (Oxoid, Hants, UK) supplemented with 0.5% glucose (GM17) and cultured at 30°C. For recombinant strains harboring plasmid vectors, erythromycin (Em) was used as a screening agent in GM17 broth and MRS broth at a final concentration of 25 μg ml^{-1}. All bacteria were stored at −80°C in their respective media with 30% (wt/vol) glycerol and cultivated with 10% inoculation more than twice before use.

DNA sequencing and genetic analysis. The nucleotide sequences surrounding two bacteriocin structural genes (*gatAX* and *gasAX*) presumed to encode GT and Acd LF221A were determined with primer walking, using the chromosomal DNA of *L. gasseri* LA327 prepared by the method of Luchansky et al. (39) as the template. The DNA sequencing was performed using the dideoxy chain termination method with a Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA) and a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocols.

Open reading frames (ORFs), promoters, and terminators on the determined DNA sequence were deduced using the GENETYX-MAC software (Genetyx Corporation, Tokyo, Japan) and an online genetic analysis site, SoftBerry (http://www.softberry.com/). Predicted amino acid sequences of peptides and proteins encoded by the DNA sequences of the ORFs were subjected to homology searching using the BLAST program in the DDBJ database (http://blast.ddbj.nig.ac.jp/top-j.html). The cellular localization and transmembrane segments (TMS) of each peptide and protein were deduced using the online program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/).

Electrotransformation of bacteria. Electrotransformation of *L. gasseri* strains, *L. lactis* subsp. *lactis* IL-1403, and *L. lactis* subsp. *cremoris* MG1363 was performed as described by Ito et al. (40) and Holo and Nes (41). Transformants were selected by using 25 μg ml^{-1} Em.

Deletion of the GT structural genes (*gatAX*) and ABC-type transporter gene (*gatT*) from *L. gasseri*. The primers used in this study are summarized in Table 9. The cloning vector, pTERM13 (40), is derived from pSY1 (GenBank accession no. E05087), which carries a replication protein gene (*repA*) identical to that of pWVO1 (42). The temperature-sensitive (ts) mutation known for pWVO1 *repA* (43) was transplanted to *repA* of pTERM13 to obtain a novel ts vector, pTERM09. In order to remove *gatAX* and *gatT* from the chromosomes of *L. gasseri* LA158 and LA327, a double-crossover (DCO) substitution was

TABLE 8 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference(s) or source
Strains		
Lactobacillus gasseri		
LA39(JCM 11657)	Producer of gassericin A	Laboratory collection
LA158(JCM 11046)	Producer of gassericin T, plasmid free	Laboratory collection
LA327	Producer of gassericin T and gassericin S, plasmid free	Laboratory collection
LA158 Δ*gatAX*	Host for recombinant plasmids, plasmid free, derivative of *Lactobacillus gasseri* LA158 lacking the *gatAX* operon	This study
LA158 Δ*gatX*	GatA producer, plasmid free, derivative of *Lactobacillus gasseri* LA158 lacking the *gatX* operon	This study
LA158 Δ*gatA*	GatX producer, *Lactobacillus gasseri* LA158 lacking the *gatA* operon	This study
LA327 Δ*gatAX*	Producer of gassericin S, plasmid free, derivative of *Lactobacillus gasseri* LA327 lacking the *gatAX* operon	This study
LA327 Δ*gatT*	Plasmid free, derivative of *Lactobacillus gasseri* LA327 lacking the *gatT* operon	This study
Lactobacillus delbrueckii subsp. *bulgaricus* JCM1002^T	Indicator for bacteriocin activity assay	JCM[b]
Lactococcus lactis subsp. *cremoris* MG1363	Intermediate host for recombinant plasmids, plasmid free, derivative of *Lactococcus lactis* subsp. *cremoris* NCDO 712	48
Lactococcus lactis subsp. *lactis* IL1403	Intermediate host for recombinant plasmids, plasmid free	49
Plasmids		
pSYE2	Em^r; pSY1 derivative with erythromycin resistance gene (*emrA*) of pAMβ1 from *Enterococcus faecalis*	50
pIL253-P_{32}	Em^r; pIL253 derivative with P_{32} promotor	51
pTERM09	Em^r; pSY1 derivative with ts mutation in the *repA* gene	40, 43
pGS-AX	Em^r; pIL253-P_{32} derivative carrying *gasAX*	This study
pGS-AXI	Em^r; pIL253-P_{32} derivative carrying *gasAXI*	This study
pGS-AXΔA	Em^r; pGS-AX derivative with *gasA* eliminated	This study
pGS-AXΔX	Em^r; pGS-AX derivative with *gasX* eliminated	This study
pGS-AXIΔA	Em^r; pGS-AXI derivative with *gasA* eliminated	This study
pGS-AXIΔX	Em^r; pGS-AXI derivative with *gasX* eliminated	This study

[a]Em^r, erythromycin resistance.
[b]JCM, Japan Collection of Microorganisms, Tsukuba, Japan.

used. The 5′-flanking 763-bp and the 3′-flanking 770-bp sequences of *gatAX*, or the 5′-flanking 792-bp and the 3′-flanking 791-bp sequences of *gatT*, were amplified and joined by using the splice-overlap extension PCR method with the primers Δ*gatAX* pr1, Δ*gatAX* pr2, Δ*gatAX* pr3, and Δ*gatAX* pr4, as well as Δ*gatT* pr1, Δ*gatT* pr2, Δ*gatT* pr3, and Δ*gatT* pr4. Similarly, *gatA* and *gatX* were removed from chromosome of *L. gasseri* LA158. After the 5′-flanking 763-bp, and the 3′-flanking 767-bp sequences of *gatA*, or the 5′-flanking 770-bp and the 3′-flanking 770-bp sequences of *gatX*, were amplified with primers Δ*gatA* pr1, Δ*gatA* pr2, Δ*gatA* pr3, and Δ*gatA* pr4, as well as Δ*gatX* pr1, Δ*gatX* pr2, Δ*gatX* pr3, and Δ*gatX* pr4, they were joined using the aforementioned method. The PCR was done using Phusion DNA polymerase (New England Biolabs, MA, USA). The joined fragment was cloned into the unique SmaI site of pTERM09 to construct pLGΔ*gatAX* and pLGΔ*gatT*. These recombinants were electrotransformed to *L. gasseri* LA158 and/or LA327, and transformants were selected at the permissive temperature of 32°C on MRS agar plates containing Em. Integration of the recombinants into the chromosomal *gat* locus of *L. gasseri* LA158 and/or LA327 was done by cultivating the transformant at 39°C, and DCO resolution of pLGΔ*gatAX* and pLGΔ*gatT* from the chromosome was induced by cultivating the integrant at 32°C in MRS medium without Em. Colony-direct PCR with Δ*gatAX* pr1 and Δ*gatAX* pr4 or Δ*gatT* pr1 and Δ*gatT* pr4 was used to screen *gatAX*- and *gatT*-deletant clones, from which 1.5-kb and 1.6-kb fragments, respectively, were amplified. Each relevant fragment was sequenced to confirm the correctness of the deletion. The *gatAX* and *gatT* deletants thus obtained were designated LA158 Δ*gatAX*, LA327 Δ*gatAX*, and LA327 Δ*gatT*.

Construction of GS-producing strains for homologous expression. Molecular cloning was performed using standard methods (44). Recombinant plasmids of *gasAX* and *gasAXI* (pGS-AX and pGS-AXI, respectively) were constructed in order to obtain the GS producers. After *gasAX* and *gasAXI* were amplified by PCR using corresponding primers (*gasA-SalI* Fw and *gasX-XbaI* Rv for *gasAX* and *gasA-SalI* Fw and *gasI-XbaI* Rv for *gasAXI*), PCR was performed using chromosomal DNA from *L. gasseri* LA327 as the template DNA, TaKaRa *Ex Taq* polymerase (EC 2.7.7.7; TaKaRa Bio Inc., Shiga, Japan), and a T100 thermal cycler (Bio-Rad Laboratories, Watford, UK). Each PCR product then was ligated with expression vector pIL253-P_{32} using T4 DNA ligase (EC 6.5.1.1; TaKaRa Bio Inc.) via double digestion using SalI and XbaI (EC 3.1.21.4; TaKaRa Bio Inc.). Furthermore, to construct the GasA and GasX producers, recombinant plasmids of *gasA*, *gasX*, *gasAI*, and *gasXI* (pGS-A, pGS-X, pGS-AI, and pGS-XI, respectively) were constructed by deletion of the *gasA* or *gasX* region from pGS-AX and pGS-AXI. Following this, inverse PCR using outward-facing primers for deletion of each region (primer pair pGSΔA Fw and pGSΔA Rv for deletion of *gasA* and primer pair pGSΔX Fw and pGSΔX Rv for deletion of *gasX*) was performed. Inverse PCR products were phosphorylated using T4 polynucleotide kinase (EC 2.7.1.78; TaKaRa Bio Inc.) and continuously

TABLE 9 Primers used in this study

Primer	Sequence (5′to 3′)	Purpose
Δ*gatA* pr1	AGTACAGTCTCTTCGGTTGG	Removing *gatA* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatA* pr2	AGCCATATTAATTCCAATAAAGACCTCCTA	Removing *gatA* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatA* pr3	TAGGAGGTCTTTATTGGAATTAATATGGCT	Removing *gatA* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatA* pr4	CTTAATTTCTGAGTTTTTCC	Removing *gatA* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatX* pr1	AAACTTTTCTGTACCTACAG	Removing *gatX* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatX* pr2	AAATCTCTATATAAAATTAATTCCCTACTT	Removing *gatX* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatX* pr3	AAGTAGGGAATTAATTTTATATAGAGATTT	Removing *gatX* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatX* pr4	CATATTTCTGAGGTGATACA	Removing *gatX* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatAX* pr2	AAATCTCTATATAAAAATAAAGACCTCCTA	Removing *gatAX* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatAX* pr3	TAGGAGGTCTTTATTTTTATATAGAGATTT	Removing *gatAX* from the chromosomes of *Lactobacillus gasseri* LA158 and LA327
Δ*gatT* pr1	AAAAAGCAAGATCCAAATGCACA	Removing *gatT* from the chromosomes of *Lactobacillus gasseri* LA327
Δ*gatT* pr2	CTTCACTAAGTCATAATATTGAATACTATT	Removing *gatT* from the chromosomes of *Lactobacillus gasseri* LA327
Δ*gatT* pr3	AATAGTATTCAATATTATGACTTAGTGAAG	Removing *gatT* from the chromosomes of *Lactobacillus gasseri* LA327
Δ*gatT* pr4	AGTTTCAGGCAATTTAAATCC	Removing *gatT* from the chromosomes of *Lactobacillus gasseri* LA327
pIL F4	CGGTTACTTTGGATTTTTGTGAG	Confirming PCR
pIL R4	TGCACTGATTGGTGTATCATTTC	Confirming PCR
gasA-SalI Fw	ACGCGTCGACCTAAATTAGTCACTTTTCCTCTTAAG	Amplifying *gasAX* and *gasAXI* located in the chromosome of *Lactobacillus gasseri* LA327
gasX-Xba Rv	GCTCTAGACTATCCATATTCCCGTCATATAC	Amplifying *gasAX* located in the chromosome of *Lactobacillus gasseri* LA327
gasI-Xba Rv	GCTCTAGAGATTATTACCAAATTGAACCTAAGAAC	Amplifying *gasAXI* located in the chromosome of *Lactobacillus gasseri* LA327
pGSΔA Fw	GAGGTGGAAGATAATGATCG	Removing *gasA* from pGS-AX and pGS-AXI
pGSΔA Rv	CTTAAGAGGAAAAGTGACTAATTTAG	Removing *gasA* from pGS-AX and pGS-AXI
pGSΔX Fw	TTGGTTCTACAAACTACTAGTGG	Removing *gasX* from pGS-AX and pGS-AXI
pGSΔX RV	TTTCGATCATTATCTTCCACCTC	Removing *gasX* from pGS-AX and pGS-AXI

self-ligated using T4 DNA ligase (TaKaRa Bio Inc.). Each recombinant plasmid was transformed into an intermediate strain, *Lactococcus lactis* subsp. *cremoris* MG1363, and then subsequently into the final expression host strain, *L. gasseri* LA158 Δ*gatAX*.

Bacteriocin activity assay by the agar well diffusion method. The CFS of each *L. gasseri* strain was prepared by centrifugation (8,000 × *g*, 10 min, 4°C) of the MRS cultures and filtrated through a 0.45-μm membrane filter (Advantec, Tokyo, Japan). Bacteriocin activity was assayed using the agar well diffusion method (45). Samples (CFSs) were twofold serially diluted using 0.85% phosphate-buffered saline (PBS). MRS agar plates (90 mm in diameter and 4 mm thick, 15 ml) containing 1.5% (wt/vol) agar (Oxoid) were overlaid with a soft agar lawn (10 ml 0.75% agar, 10 μg ml^{-1} Em) and inoculated with a one-tenth-diluted overnight culture (250 μl) of the indicator strain *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2). Wells (6 mm in diameter) were cut off from the plates, and then 65 μl of the twofold serially diluted (1:1 to 1:1,023) samples was added to each well. The plates were incubated for 18 h at 37°C. The unit of bacteriocin activity (arbitrary unit [AU]) was defined as the reciprocal of the highest dilution inhibiting the growth of the indicator strain. The assay for each sample was performed at least in triplicate.

Bacteriocin activity assay of Gas and Gat producers and verification of synergistic activities of GS and/or GT. Bacteriocin activities of Gas producers were assayed by the agar well diffusion method as described above. After the bacteriocin activities in the CFSs of GasAX and GasAXI producers were tested, those of GasA, GasX, GasAI, and GasXI producers were examined alone and in combination (GasA or -AI and GasX or -XI, 1:1), and then the activities of Gat producers (producing GatA and/or GatX) were similarly assayed. Furthermore, the CFSs of GatA, GatX, GasA, and GasX producers were subjected to the assay in combination (six patterns of two peptides and the combination of four peptides).

In order to verify the synergistic activities of GS (GasA plus GasX), GT (GatA plus GatX), and GS (GasA plus GasX) plus GT (GatA plus GatX), isobologram assays were performed with the minimum concentration of the CFS of each bacteriocin peptide, which shows the inhibitory zone on the agar well diffusion assay, and the concentration of each undiluted (original) CFS (65 μl) was defined as 1.00.

Preparation of crude GS and *in situ* activity assay. The CFS of the GS producer *L. gasseri* LA158 Δ*gatAX*(pGS-AXI), was concentrated approximately 40 times by ultrafiltration (Amicon Ultra-100K centrifugal filter units; Merck Millipore, Tullagreen, Ireland), and then the concentrate (crude GS) was subjected to SDS-PAGE following Laemmli's method (46) with a 4.5% spacer gel and 20% separating gel. The Amersham ECL Rainbow low-range marker (GE Healthcare, Tokyo, Japan), with marker range of 3,500 to 40,000 Da, was used as a molecular marker. For detection of bacteriocin activity on the gel, an *in situ* activity assay was performed as described by Daba et al. (47). Briefly, the half-cut gel was put in a petri dish after immobilization with 20% isopropanol–10% acetate and was washed with Milli-Q water; MRS agar containing the indicator strain was then stratified and incubated at 37°C for 24 h. The bacteriocin activity of GS was observed as a clear zone, and the molecular mass was estimated by electrophoretic mobility.

Assay of pH stability of GS. The pHs of the CFSs from the GS producer *L. gasseri* LA158 Δ*gatAX* (pGS-AXI) and the non-bacteriocin producer (control) *L. gasseri* LA158 Δ*gatAX* were adjusted to 2, 4, 7, and 10 with 1 N HCl and 1 N NaOH at room temperature. Following this, the bacteriocin activities of these CFSs were assayed by the agar well diffusion method, using *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T(pSYE2) as the indicator.

Assay of heat stability of GS. The CFS of the GS producer *L. gasseri* LA158 Δ*gatAX*(pGS-AXI) was dispensed at 100 μl in a 0.2-ml PCR tube (Bio-Rad) and autoclaved (121°C for 15 min) or heated (95°C for

5 min and 70°C for 1 h). For confirmation of the preservation stability, CFS containing GS was incubated at 37°C and 4°C for 1 month and then utilized in the bacteriocin activity assay.

Assay of proteolytic sensitivity of GS. Protease solutions (0.2%, wt/vol) were prepared by dissolving pepsin (EC 3.4.23.1) (Wako Pure Chemical Industries, Osaka, Japan) in 0.2 M HCl-KCl buffer (pH 2) and dissolving trypsin (4,200 USP units/mg; EC 3.4.21.4) (Wako), alpha-chymotrypsin (40 to 50 units/mg; EC 3.4.21.1) (MP Biomedicals, Illkirch, France), and proteinase K (590 units/ml; EC 3.4.21.64) (Wako) in 0.2 M sodium phosphate buffer (pH 8). The CFSs of the GA producer (*L. gasseri* LA39), the GT producer (*L. gasseri* LA158), and the GS producer [*L. gasseri* LA158 Δ*gatAX*(pGS-AXI)] were mixed with each protease solution in an equal amount and were incubated at 37°C for 5 h in a water bath. These treated CFSs were utilized in the bacteriocin activity assay.

Accession number(s). The 6,935-bp and 1,143-bp DNA sequences containing nine genes (*gat* operon) and three genes (*gas* operon), respectively, were deposited in the DDBJ database under accession numbers LC389592 and LC389591, respectively.

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