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Novel Group of Leaderless Multipeptide Bacteriocins from Gram-Positive Bacteria

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ABSTRACT

From raw milk we found 10 *Lactococcus garvieae* isolates that produce a new broad-spectrum bacteriocin. Though the isolates were obtained from different farms, they turned out to possess identical inhibitory spectra, fermentation profiles of sugars, and repetitive sequence-based PCR (rep-PCR) DNA patterns, indicating that they produce the same bacteriocin. One of the isolates (*L. garvieae* KS1546) was chosen for further assessment. Purification and peptide sequencing combined with genome sequencing revealed that the antimicrobial activity was due to a bacteriocin unit composed of three similar peptides of 32 to 34 amino acids. The three peptides are produced without leader sequences, and their genes are located next to each other in an operon-like structure, adjacent to the genes normally involved in bacteriocin transport (ABC transporter) and self-immunity. The bacteriocin, termed garvicin KS (GarKS), showed sequence homology to four multipeptide bacteriocins in databases: the known staphylococcal aureocin A70, consisting of four peptides, and three unannotated putative multipeptide bacteriocins produced by *Bacillus cereus*. All these multipeptide bacteriocin loci show conserved genetic organization, including being located adjacent to conserved genetic determinants (Cro/cI and integrase) which are normally associated with mobile genetic elements or genome rearrangements. The antimicrobial activity of all multipeptide bacteriocins was confirmed with synthetic peptides, and all were shown to have broad antimicrobial spectra, with GarKS being the most active of them. The inhibitory spectrum of GarKS includes important pathogens belonging to the genera *Staphylococcus*, *Bacillus*, *Listeria*, and *Enterococcus*.

IMPORTANCE

Bacterial resistance to antibiotics is a very serious global problem. There are no new antibiotics with novel antimicrobial mechanisms in clinical trials. Bacteriocins use antimicrobial mechanisms different from those of antibiotics and can kill antibiotic-resistant bacteria, but the number of bacteriocins with very broad antimicrobial spectra is very small. In this study, we have found and purified a novel three-peptide bacteriocin, garvicin KS. By homology search, we were able to find one known and three novel sequence-related bacteriocins consisting of 3 or 4 peptides. None of the peptides has modified amino acids in its sequence. Thus, the activity of all bacteriocins was confirmed with chemically synthesized peptides. All of them, especially garvicin KS, have very broad antibacterial spectra, thus representing a great potential in antimicrobial applications in the food industry and medicine.

Bacterial resistance to antibiotics has become a serious worldwide problem (1). In spite of that, only two new classes of antibiotics—oxazolidinones (linezolid) and cyclic lipopeptides (daptomycin)—have reached the market during the last 3 decades (2), and development of resistance against these antibiotics has been reported (3). The situation is getting worse, as there are no new antibiotic classes in phase II or III clinical trials and none in the preregistration stage (4). Consequently, there is an urgent need for new antimicrobial agents with different killing mechanisms and new strategies to overcome multidrug-resistant bacteria (5).

One alternative to antibiotics is a diverse group of antimicrobial peptides called bacteriocins (6). In Gram-positive (G^+) bacteria, most bacteriocins are small, ribosomally synthesized peptides able to inhibit growth or kill other G^+ bacteria in competition for nutrients or habitats. Most bacteriocins have narrow inhibitory spectra, targeting species or genera closely related to the producer (7), but some have wide inhibitory spectra (8). Unlike most antibiotics, which are enzyme inhibitors, bacteriocins are membrane-active antimicrobials; i.e., they act by disrupting the membrane integrity of sensitive cells, causing leakage of intracellular solutes and eventually cell death (9). Thus, due to different modes of action, bacteriocins are normally active against both antibiotic-sensitive pathogens and their antibiotic-resistant counterparts. To date, the best-studied bacteriocins are from lactic acid bacteria (LAB) because of their status as generally recognized as safe (GRAS) for human consumption. These bacteriocins can be safely used as natural preservatives in foods and drinks (10). However, so far only two bacteriocins, both with broad antimicrobial spectra, nisin and pediocin PA-1, have been autho-

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rized as preservatives in the food industry (11). The need for new bacteriocins with broad antimicrobial spectra is still great (12).

Generally, the heat-stable bacteriocins from Gram-positive bacteria are divided into two major classes: class I (lantibiotics) and class II (nonlantibiotics). Lantibiotics are small peptides, of 19 to 38 amino acids, containing posttranslationally modified residues such as lanthionine or β-methyllanthionine (6). Class II bacteriocins contain peptides without modified residues or with minor modifications (e.g., disulfide bridge or circularization) and are divided further into four subclasses. Subclass IIa contains medium-length bacteriocins (37 to 48 residues) with strong antilisterial activity. They are often called pediocin-like bacteriocins after pediocin PA-1, the first member of the class (13). All members of the group contain a conserved N-terminal sequence—the YGNGV "pediocin box"—and one or two intrachain disulfide bonds (14). Subclass IIb consists of two-peptide bacteriocins whose antimicrobial activity relies on the complementary action of the two different peptides (15). Subclass IIc consists of circular bacteriocins with N- to C-terminal covalent linkage. Their circular nature makes the peptides extremely resistant to environmental abuses as well as to many proteolytic enzymes (16). Subclass IId is a miscellaneous group encompassing bacteriocins that do not fit into the three other groups. This group is relatively diverse in terms of amino acid sequence, structure, mechanisms of secretion, and action. It also includes a leaderless bacteriocin subgroup whose members are different from most bacteriocins in that they do not involve an N-terminal leader sequence for export (6).

Dairy products are common sources for bacteriocins; species of *Lactococcus* are often prevalent as active producers. We have recently performed a large and systematic survey on the microbial quality of raw bovine milk from many different farms in Kosovo, and a large collection (over 1,800 isolates) of LAB has been isolated (17). In the present work, we used this collection to screen for bacteriocin producers. We describe here the screening assay, purification, and identification of a novel and broad-inhibitory spectrum bacteriocin with potent activity against many important pathogens. It is a multipeptide leaderless bacteriocin, produced by an isolate of *Lactococcus garvieae*. We also performed genome sequencing to identify the genes involved in bacteriocin synthesis. By homology search we were able to identify three unannotated sequence-related multipeptide bacteriocins produced by *Bacillus cereus* and to prove their activity. Based on this, we propose a separate subgroup for these multipeptide bacteriocins due to their related biochemical composition and genetic organization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial collection of LAB which was used in the screening assay was from raw bovine milk samples collected from 221 farms in Kosovo from November 2011 to June 2012 (17). Cells from the collection and the indicator strains (see below) were routinely grown in brain heart infusion (BHI) (Oxoid, United Kingdom) broth at 30°C under aerobic conditions without shaking.

Screening for broad-spectrum bacteriocin producers. To screen for wide-inhibition-spectrum bacteriocin producers, strains of *Lactococcus lactis*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Listeria innocua*, and *Staphylococcus aureus* were used as indicators in the first round of screening. The antimicrobial screening was performed using the agar diffusion bioassay as previously described (18). Briefly, indicator cells from overnight cultures were diluted 100-fold in 5 ml of BHI soft agar and plated out as a lawn on BHI agar plates. Potential bacteriocin producers at volumes of 3 μl were spotted on the indicator lawn and then incubated at 30°C for 24 h for cell growth and cell inhibition. Inhibition was detected as clear zones around the spotted cells.

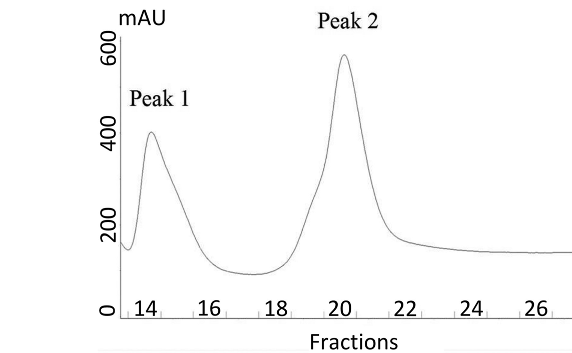

FIG 1 Elution profile of garvicin KS with 2-propanol in the first RPC. Peaks 1 and 2 represent fractions with antimicrobial activity.

For protease sensitivity, 2 μl of proteinase K (Sigma-Aldrich) at 20 μg/ml was applied near the spotted cells. Sensitivity was seen when indicator cell growth was not affected in the region close to where proteinase K had been applied. Heat sensitivity was assessed at 100°C for 5 min before samples were tested for bacteriocin activity.

DNA technologies. Total genomic DNA was isolated by using FastPrep (Bio101/Savant) and DNA minikit (Omega Bio-tek Inc., GA). Amplification of the 16S rRNA gene by PCR was carried out using the primers 5F (5′-GGTTACCTTGTTACGACTT-3′) and 11R (5′-TAACACATGCA AGTCGAACG-3′) as previously described (19). PCR products were purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and sent to GATC Biotech, Germany, for sequencing. For genetic fingerprinting, repetitive sequence-based PCR (rep-PCR) was performed using oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) and a protocol previously described (20). Amplicons were visualized under UV light after electrophoretic migration through a 1.0% agarose gel. The whole-genome sequencing service was provided by Norwegian Sequencing Center (University of Oslo, Oslo, Norway). Quality-filtered reads were assembled into contigs using CLC Genomics workbench 5.5 (CLC Inc., Aarhus, Denmark) as previously described (21). Genome annotation was performed using the RAST (Rapid Annotation using Subsystem Technology) server (22).

API test-fermentation profiling. Carbohydrate fermentation was determined by using the API 50CH test according to the manufacturer's instructions (bioMérieux SA, France).

Bacteriocin purification and assay. The bacteriocin-producing strain *L. garvieae* KS1546 was grown in M17 medium (Oxoid) supplemented with 0.5% (wt/vol) glucose at 30°C without shaking. Purification was done as described by Holo et al. (18). The bacteriocin was purified from a 1-liter culture. The cells were grown to the early stationary phase and removed by centrifugation at 10,000 × *g* for 15 min at 4°C. The bacteriocin was precipitated from the culture supernatant with ammonium sulfate (45% saturation at 4°C) and harvested by centrifugation (15,000 × *g* and 4°C for 30 min). The protein pellet containing the crude bacteriocin was dissolved in 100 ml of water containing 0.1% (vol/vol) trifluoroacetic acid (TFA; Sigma-Aldrich) (buffer A). The sample was applied on a HiPrep 16/10 SP-XL column (GE Healthcare Biosciences) equilibrated with buffer A. The column was washed with 100 ml of 20 mM sodium phosphate buffer at pH 6.8 before elution of the bacteriocin with 50 ml of 0.2 M NaCl. The eluate was applied to a Resource reverse-phase chromatography (RPC) column (1 ml) (GE Healthcare Biosciences) connected to an ÅKTA purifier system (Amersham Pharmacia Biotech). A linear gradient of isopropanol (Merck) with 0.1% (vol/vol) TFA (buffer B) at a flow rate of 1.0 ml min^{-1} was used for elution. The crude bacteriocin was eluted in two peaks with 31 and 34% buffer B, respectively (Fig. 1). Since the second (34% of isopropanol) peak fractions were about 16 times more

active, this peak was chosen for further purification. Active fractions of the second peak were diluted in buffer A five times and applied on an RPC C_8 column (Amersham Biosciences). Bacteriocin peptides were eluted with 36% buffer B. Fractions showing antibacterial activity were chosen for mass spectrometry (MS) analysis.

Bacteriocin activity was determined using a microtiter plate assay (18). The plates were incubated at 30°C for 8 h, and growth was measured spectrophotometrically at 600 nm (A_{600}) at 15-min intervals using SPECTROstarNano (BMG LABTECH, Germany). The MIC was defined as the bacteriocin concentration (bacteriocin units [BU] per ml) that inhibited the growth of the indicator strain by at least 50% in 200 μl of culture (i.e., 50% of the turbidity of the control culture without bacteriocin).

MS analysis and N-terminal amino acid sequencing. Acquisition of MS data was performed on an Ultraflex MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) instrument operated in reflection mode with delayed extraction. Positively charged ions in the *m/z* range of 200 to 6,000 were analyzed using an acceleration voltage of 25 kV. The sample spectra were calibrated externally with a calibration standard covering the *m/z* range from 700 to 3,100 (Bruker Daltonics, Bremen, Germany). Two of the most active fractions after the second RPC step (C_8 column) were chosen for N-terminal amino acid sequencing by Edman degradation using ABI Procise 494 sequencer (Alphalyse, Denmark).

Synthetic peptides. All the peptides were synthesized by Pepmic Co., Ltd., China, with 90 to 99% purity, except for CehA, CehB, CexA, and CevA (85% purity) due to technical difficulties (poor purification). All the synthesized peptides were not formylated. The peptides were solubilized to concentrations of 0.1 to 2 mg/ml in 0.1% (vol/vol) trifluoroacetic acid and stored at −20°C until use.

Accession number(s). The sequence for the garvicin KS DNA locus has been submitted to GenBank under accession number KU821057.

RESULTS

Screening for broad-inhibitory-spectrum bacteriocin producers. In a previous study of microbial quality of raw bovine milk in Kosovo, a large collection (1,854 isolates) of LAB was created (17). We used this collection to screen for broad-inhibitory-spectrum bacteriocin producers using a panel of five indicators: *Lactococcus lactis*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Listeria innocua*, and *Staphylococcus aureus*. Of the 1,854 isolates, 107 were active against all five indicators.

Lactococcus species are frequently found in raw milk and dairy products, and many of them are known as bacteriocin producers. To avoid identification of known lactococcal bacteriocins such as nisin and lactococcins G, A, B, and M, we used producers of these bacteriocins as indicators for the next round of screening. Our assumption is that these known bacteriocin-producing indicators will be immune to their own bacteriocins (23). Among the 107 isolates, only 10 were found to be capable of killing all these producers (data not shown). The 10 isolates were genotyped by 16S rRNA gene sequencing and showed 100% sequence identity to *L. garvieae*.

Characterization of the 10 bacteriocin-producing *L. garvieae* isolates. The 10 *L. garvieae* isolates were from 10 different farms in 4 geographically different Kosovo regions (17). Their antimicrobial activity was heat stable and proteinase sensitive, properties typical of bacteriocins. They had identical inhibition spectra against 46 Gram-positive bacteria from 8 different genera (data not shown), indicating that they might produce the same bacteriocin(s). rep-PCR showed that all 10 *L. garvieae* isolates had the same pattern of amplified DNA bands (data not shown), and API 20E tests showed that the 10 isolates shared the same profile of fermentation of different sugars (see Table S1 in the supplemental material). Based on that, we reckoned that they all were probably very similar genetically and produced the same antimicrobial activity. Therefore, only one of the bacteriocin producers, termed *L. garvieae* KS1546, was chosen for bacteriocin purification.

Purification and characterization of the bacteriocin activity. Purification was accomplished by established methods for bacteriocins, including cation-exchange chromatography followed by two reverse-phase chromatography (RPC) steps. In the first RPC step, two peaks of antimicrobial activity were identified that corresponded with peaks absorbance at 215 nm (Fig. 1). The first peak of activity was eluted with 31% 2-propanol and the second with 34%, indicating the presence of molecules with different levels of hydrophobicity. MS analysis of the peaks' fractions revealed several predominant masses from about 3,000 to 3,500 Da (Fig. 2) in both of them. Fractions from both peaks were active against *L. lactis*, *L. innocua*, *S. aureus*, *L. sakei*, and *L. plantarum* (data not shown). Peak 2 had antimicrobial activity about 16 times higher than that of peak 1 and was rechromatographed.

The second RPC active fractions were obtained with 36% 2-propanol; however, significant loss of bacteriocin activity was observed, as the final yield was only 0.3% of the starting activity (Table 1). MS analysis of two most active fractions of second RPC showed that both contained a predominant 3,478.6-Da peptide (data not shown). Subsequent N-terminal amino acid sequencing by Edman degradation revealed identical amino acid sequences of 20 residues in both fractions: MGAIIKAGAKIVGKGVLGGG.

Identification of the multiple peptides and the bacteriocin-encoding operon. In a separate work, the genome of *L. garvieae* KS1546 was sequenced (unpublished data). Based on the amino acid sequence obtained, we searched for the corresponding DNA sequence in the bacterial genome of the producer. An open reading frame (ORF) was found to encode a peptide of 34 amino acid residues, of which the first 20 N-terminal amino acids perfectly matched the peptide sequence obtained by the Edman degradation (Table 2). However, the theoretical monoisotopic mass of the gene-derived peptide was 3,450.9 Da, which is 28 Da less than the mass determined by the MS analysis of the purified peptide (3,478.6 Da). Further analysis of the flanking regions revealed two additional small ORFs that encoded putative peptides with high sequence similarity to the aforementioned ORF. The additional two peptides were of 34 and 32 amino acids, with theoretical monoisotopic masses of 3,158.8 and 3,097.7 Da, respectively (Table 2). Interestingly, these two masses were also about 28 Da smaller than the two for the predominant peaks (3,186.6 and 3,125.5 Da) identified in the MS analysis of peak 2 (Fig. 2B). The three putative bacteriocin-like ORFs were named *gakA*, *gakB*, and *gakC* in the order in which they are aligned in the DNA.

Confirmation of the bacteriocin activity with chemically synthesized peptides. The three identified peptides termed GakA, GakB, and GakC (Tables 2 and 3) were chemically synthesized and tested for antimicrobial activity against *L. lactis* IL1403. The individual peptides had poor activity, with MICs of 360 nM for GakA and 6 μM for GakC and GakB having no measurable activity at the highest concentration tested (12 μM). However, when the three peptides were combined at equal molar concentrations, the MIC of the mixture decreased drastically, to 10 nM. Combinations of any two peptides did not show any increased antimicrobial activity over that of the individual peptides (data not shown). These results strongly indicate that the bacteriocin unit, here called garvicin KS (GarKS), is a multipeptide bacteriocin composed of the three peptides GakA, GakB, and GakC.

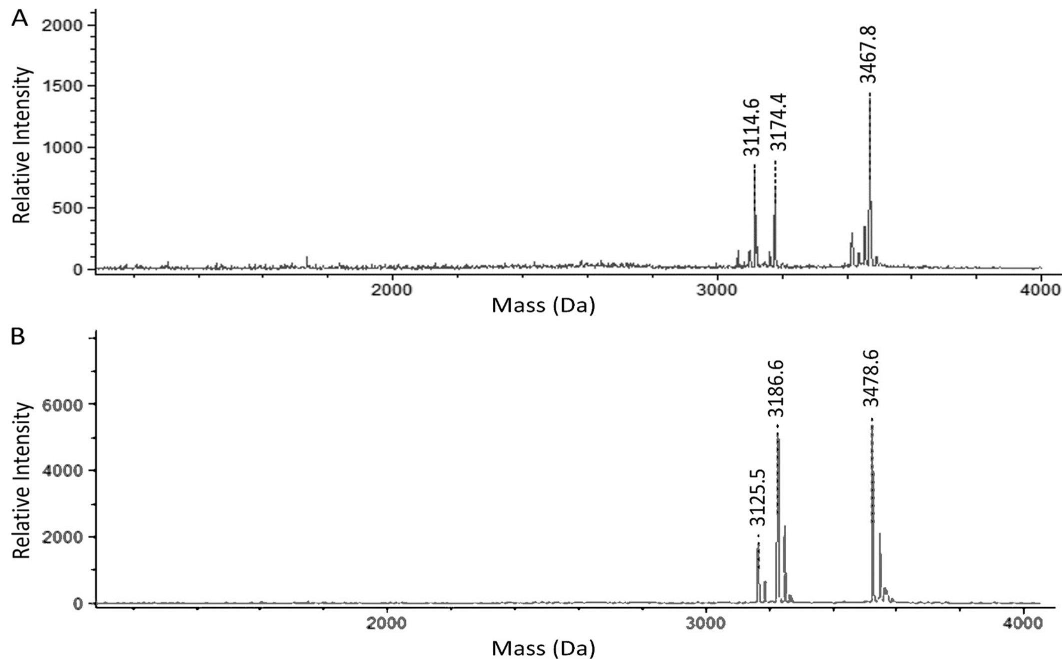

FIG 2 Mass spectrometry analysis of active fractions of garvicin KS after the first RPC. (A) Peak 1; (B) peak 2.

Bioinformatics search for GarKS-related ORFs in the sequence databases. By using the peptide sequences of GarKS, we performed a protein BLAST search in the bacterial data banks. Three homologues—putative leaderless bacteriocins—and one known bacteriocin were identified (Table 2). The known bacteriocin is a four-peptide bacteriocin, aureocin A70 (AurA70), produced by *S. aureus* (24). The other three putative bacteriocins were identified in the different genomes of *B. cereus* and comprised of three or four peptides. All their peptides were annotated as hypothetical proteins. The three putative bacteriocins from *Bacillus* isolates were named cereucin X (CerX; three peptides), cereucin H (CerH; four peptides), and cereucin V (CerV; three peptides) (Table 2). As can be seen from Table 2 and Fig. 3, GakA is very similar to the CexA, CehA, CevA, and CehB peptides. GakB is even more similar to the CexC, CehC, AurB, and CevB peptides.

The peptides comprising aureocin A70 and the three putative *B. cereus* bacteriocins were chemically synthesized (Table 2). As demonstrated with GarKS, the new *B. cereus* bacteriocins were highly active only when all peptides were combined. When the peptides were assessed individually, little, if any, activity was seen (Table 3). To examine and compare their inhibitory spectra, these bacteriocins were tested against a panel of 43 bacteria of different species and genera; they showed high antimicrobial activity (Table 4). Among the bacteriocins, garvicin KS was the most potent.

TABLE 1 Purification of garvicin KS

Fraction	Vol (ml)	Total activity (10^4 BU)	Yield (%)
Culture supernatant	1,000	63	100
Ammonium sulfate precipitate	100	51	81
Cation-exchange chromatography	50	26	41
Reverse-phase chromatography			
HiPrep[a]	5	10	16
C_8	5	0.2	0.3

[a] The activity shown is from peak 2 in Fig. 1.

CerH: three- or four-peptide bacteriocin? The DNA sequence suggests that CerH consists of four peptides (Table 2; Fig. 4). Surprisingly, two of them, CehA and CehB, are very similar at their C termini (Table 2). We were therefore interested to know whether one of these is dispensable in terms of constituting the bacteriocin activity. We compared the activities of these combinations: CehACD, CehBCD and CehABCD. It turned out that CehACD and CehBCD were equally active only against *B. subtilis* LMGT 3131 and some *S. aureus* strains but, when tested against the rest of the strains, the CehBCD mixture was up to 30 times more active than the CehACD mixture (Table 4). CehBCD was generally even more active than CehABCD. These observations suggest that CehA might be redundant for optimal bacteriocin activity, as it performs more poorly than CehB when combined with the other two peptides (CehCD) and also because it can be replaced by CehB without a loss of activity.

Comparison of bacteriocin loci. DNA sequences of CerH, CerV, CerX, and AurA70 loci were taken from the NCBI database (GenBank accession numbers AHDX01000055.1, AHFF01000058.1, AHCW01000073.1, and AF241888.2, respectively) to compare to that of GarKS. In addition to bacteriocin structural genes, one putative bacteriocin ABC transporter gene was found in each bacteriocin locus (Fig. 4). Moreover, GarKS, CerH, and CerX loci contain an ORF encoding a protein of 150 to 156 residues which was found to share high similarity with the AurA70 immunity protein (25) (Fig. 4 and 5). Interestingly, some other genes in these bacteriocin loci were also very similar: at the protein level, the genes encoding mercury resistance proteins in the loci of CerX and CerV were 93% identical to each other. The same level of identity was found between the integrases in the CerH and CerX loci. The genes encoding the prophage Cro/cI

TABLE 2 Sequences of GarKS and related bacteriocin peptides

Bacteriocin	Peptide	Sequence	M_w (monoisotopic)	pI
GarKS	GakA	MGAIIKAGAKIVGKGVLGGGASWLGWNVGEKIWK	3,450.9	10.18
	GakB	MGAIIKAGAKIIGKGLLGGAAGGATYGGLKKIFG	3,158.8	10.30
	GakC	MGAIIKAGAKIVGKGALTGGGVWLAEKLFGGK	3,097.7	10.18
AurA70	AurA	MGKLAIKAGKIIGGGIASALGWAAGEKAVGK	2,922.6	10.18
	AurB	MGAVAKFLGKAALGGAAGGATYAGLKKIFG	2,795.5	10.18
	AurC	MGALIKTGAKIIGSGAAGGLGTYIGHKILGK	2,952.7	10.18
	AurD	MGAVIKVGAKVIGWGAASGAGLYGLEKIFKK	3,084.8	10.00
CerV	CevA	MGAVVKGGLKIIGGTAASWLGWEAGTRIWK	2,974.6	10.28
	CevB	MGAAVKMLGKAFAGGVAGGATYGGLKKIFG	2,827.5	10.18
	CevC	MGAVVKGALKIIGGGAASGGAVYGLERIFGR	3,112.7	10.29
CerX	CexA	MGKKIGKWIITGAAGWAGWEIGEGIWK	2,942.5	9.52
	CexB	MKYLGTLIKGAAGGAGAYVGEKIYNWYKN	3,135.6	9.52
	CexC	MGALFKAALKAAGGGAAGGATYGGLKHFFG	2,796.4	10.00
CerH	CehA	MAKIGKWVVKGAAGYLGWEIGEGIWK	2,846.5	9.40
	CehB	MGALVKGGLKLIGGTAASWLGWEAGERVWK	3,140.7	9.70
	CehC	MGAIIKGGLKLVGGGAAGGFTYGGLKKIFG	2,837.6	10.18
	CehD	MGAIIKGAAKVLGKGAATGGVIYGLEKLFR	2,988.7	10.17

family proteins of CerX and CerH were even 100% identical and shared 62% and 49% identity with AurA70 and GarKS Cro/cI genes, respectively.

W26 is crucial for antimicrobial activity of GakA peptide as well as for GarKS bacteriocin. GakA is a very hydrophobic peptide and difficult to synthesize and purify, probably due to the presence of three tryptophan residues in the C-terminal half. The purity of the synthetic peptide in our experiment was at best only 90%. As an attempt to improve purification, we reduced the hydrophobicity of GakA by replacing its tryptophan residues at the C-terminal end one by one with alanine (W23A, W26A, and W33A). As expected, the modified peptides all attained higher purity (95%). However, when assessed for antimicrobial activity against *L. lactis* IL1403, W26A could not restore activity either as a single peptide or when mixed with GakB and GakC. W23A and W33A peptides were found to be about two times less active (MIC = 0.72 mM) than the wild-type peptide when assessed alone or as a mixture with GakB and GakC (MIC = 20 nM).

TABLE 3 MICs of single peptides and their combinations against *L. lactis* IL1403

Bacteriocin	Peptide	MIC (μM)	MIC of peptide mixture (nM)
Gak	A	0.36	
	B	>12	10
	C	6	
Aur	A	2	
	B	>6	95
	C	>6	
	D	>6	
Cev	A	1.3	
	B	>5	80
	C	5	
Cex	A	>6	
	B	>6	45
	C	>6	
Ceh	A	>2.5	
	B	2.5	46
	C	>2.5	
	D	5	

DISCUSSION

In this study, we aimed to isolate novel bacteriocins with broad inhibition spectra. To achieve this we employed a set of several indicators of distantly related genera (*Listeria*, *Lactobacillus*, *Lactococcus*, and *Staphylococcus*) in our initial screening. Further, by using a second set of indicators of known bacteriocin producers that are frequently found in the same or similar environments (dairy environment in our study), we were able to reduce the number of potential new broad-spectrum bacteriocins drastically,

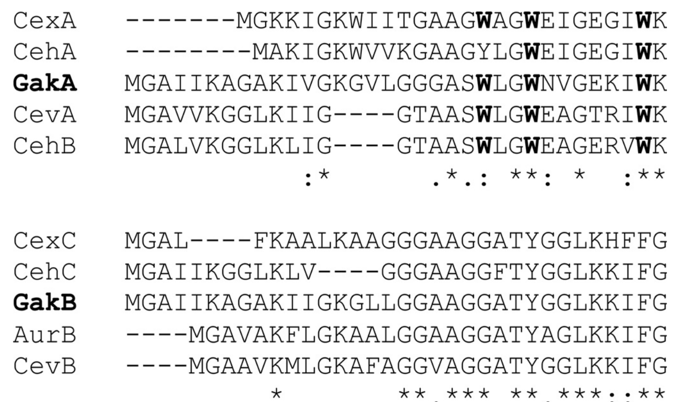

FIG 3 Clustal Omega alignment of GakA and GakB homologues peptides. Tryptophan residues at C termini are shown in bold. Stars indicate identical amino acids, colons very similar amino acids, and dots periods similar amino acids.

TABLE 4 MICs of GarKS, AurA70, CerV, CerX, and CerH against different bacterial species

Indicator strain	MIC (nM)						
					CerH[a]		
	GarKS	AurA70	CerV	CerX	A–D	A, C, D	B, C, D
S. aureus							
LMGT 3310	160	1,500	5,000	6,000	1,500	3,000	1,500
LMGT 3264	320	6,000	5,000	6,000	3,000	6,000	1,500
LMGT 3260	2,500	>6,000	>5,000	6,000	3,000	3,000	3,000
LMGT 3266	630	750	5,000	1,500	1,500	1,500	1,500
LMGT 3305	1,250	1,500	5,000	3,000	1,500	1,500	1,500
LMGT 3258	2,500	3,000	5,000	3,000	3,000	3,000	3,000
LMGT 3289	2,500	3,000	5,000	3,000	3,000	3,000	3,000
LMGT 3272	2,500	1,500	5,000	1,500	3,000	3,000	3,000
S. epidermidis LMGT 3026	320	3,000	2,500	1,500	750	1,500	1,500
E. faecalis							
LMGT 3199	160	6,000	2,500	3,000	1,500	6,000	730
LMGT 3330	160	6,000	2,500	3,000	740	3,000	730
LMGT 3359	320	6,000	5,000	6,000	3,000	6,000	1,500
LMGT 3333	160	6,000	5,000	6,000	1,500	3,000	1,500
LMGT 3143	320	6,000	5,000	6,000	3,000	6,000	1,500
LMGT 3351	160	3,000	2,500	1,500	740	1,500	730
LMGT 3200	320	6,000	2,500	1,500	1,500	3,000	730
E. faecium							
LMGT 3108	160	6,000	2,500	3,000	1,500	3,000	730
LMGT 3104	80	3,000	2,599	1,500	740	1,500	370
LMGT 2722	160	3,000	2,500	1,500	740	6,000	730
LMGT 2787	160	>6,000	3,000	1,500	370	6,000	730
LMGT 2783	320	>6,000	5,000	3,000	1,500	3,000	1,500
E. durans LMGT 3191	160	3,000	1,269	1,500	740	3,000	730
P. pentosaceus LMGT 2001	40	185	315	45	92	190	45
L. garvieae							
LMGT 1546[b]	6,000	370	5,000	1,500	740	1,500	730
LMGT 3390	40	745	1,300	740	370	1,500	370
LMGT 2217	80	3,000	5,000	3,000	370	3,000	370
L. lactis							
IL1403	10	95	80	45	46	380	22
LMGT 2084	40	370	315	90	185	760	90
LMGT 2095	5	185	160	25	23	50	10
LMGT 2057	2	45	80	3	10	90	3
LMGT 2233	10	95	160	45	25	380	10
L. sakei							
LMGT 2334	40	740	630	670	370	1,500	180
LMGT 3353	160	1,500	1,260	740	370	1,500	370
L. plantarum LMGT 2329	320	6,000	2,500	6,000	3,000	3,000	1,500
B. subtilis LMGT 3131	320	745	315	370	370	380	370
B. cereus							
LMGT 2805	80	370	315	45	90	190	45
LMGT 2731	160	370	630	45	370	380	180
LMGT 2711	80	370	315	23	92	380	45
LMGT 2735	320	1,500	630	185	185	1,500	180
L. monocytogenes							
LMGT 319	160	745	1,260	370	370	750	370
LMGT 2605	160	370	1,260	1,500	370	3,000	370
L. innocua							
LMGT 2785	160	745	2,500	1,500	1,500	3,000	730
LMGT 2710	160	745	1,260	370	370	750	370

[a] CerH was assessed in three different combinations: all peptides together (A to D) and three-peptide combinations: (i) A, C, and D and (ii) B, C, and D.
[b] GarKS producer.

from 107 to 10. These 10 producers were subsequently shown to be very similar isolates of *Lactococcus garvieae*.

L. garvieae is a LAB mostly known as a human opportunistic and a major fish pathogen (26, 27), but this species is also commonly found in milk and dairy products (17). So far, four bacteriocins have been found in different *L. garvieae* strains. Garviecin L1-5 is a small bacteriocin, with a molecular mass of about 2.5 kDa, produced by *L. garvieae* L1-5 isolated from a raw cow's milk sample. It inhibits bacteria from the *Lactococcus*, *Listeria*, *Enterococcus*, and *Clostridium* genera (28). Garvicin L1-5 has not been characterized at the protein and genetic level, so its amino acid sequence is not known. The circular bacteriocin garvicin ML, with molecular mass of 6 kDa, consists of 60 residues. It is produced by *L. garvieae* DCC43 isolated from mallard duck intestines (29). It also has a broad antimicrobial spectrum and probably is the best studied among all *L. garvieae* bacteriocins so far (30, 31). Garvieacin Q consists of 50 amino acids (5.3 kDa) and is produced by strain BCC 43578, isolated from fermented pork sausage. It is active primarily against closely related bacteria (32). Garvicin A is a 43-residue class IId bacteriocin produced by *L. garvieae* 21881 (human clinical isolate) with a mass of 4.7 kDa. It has a narrow antimicrobial spectrum (33). Garvicin KS, the bacteriocin identified in the present study, is different from the aforementioned bacteriocins in composition. It is a multipeptide bacteriocin composed of three small similar peptides with a size between 32 and 34 amino acids.

Purification of garvicin KS was carried out by cation-exchange chromatography followed by two steps of RPC. The active fractions after the second RPC showed a 30-fold loss of activity. The reason for that activity decrease became clear after the analysis of the bacteriocin DNA locus, which showed that garvicin KS consists of three structural genes. The residual activity after the purification in the fractions used for amino acid sequence was therefore likely due to GakA alone or to GakA contaminated with trace amounts of GakB and GakC that together restored the observed activity. Such contamination is in fact relatively common during purification of bacteriocin peptides from multiple bacteriocin producers because these peptides often share very similar physi-

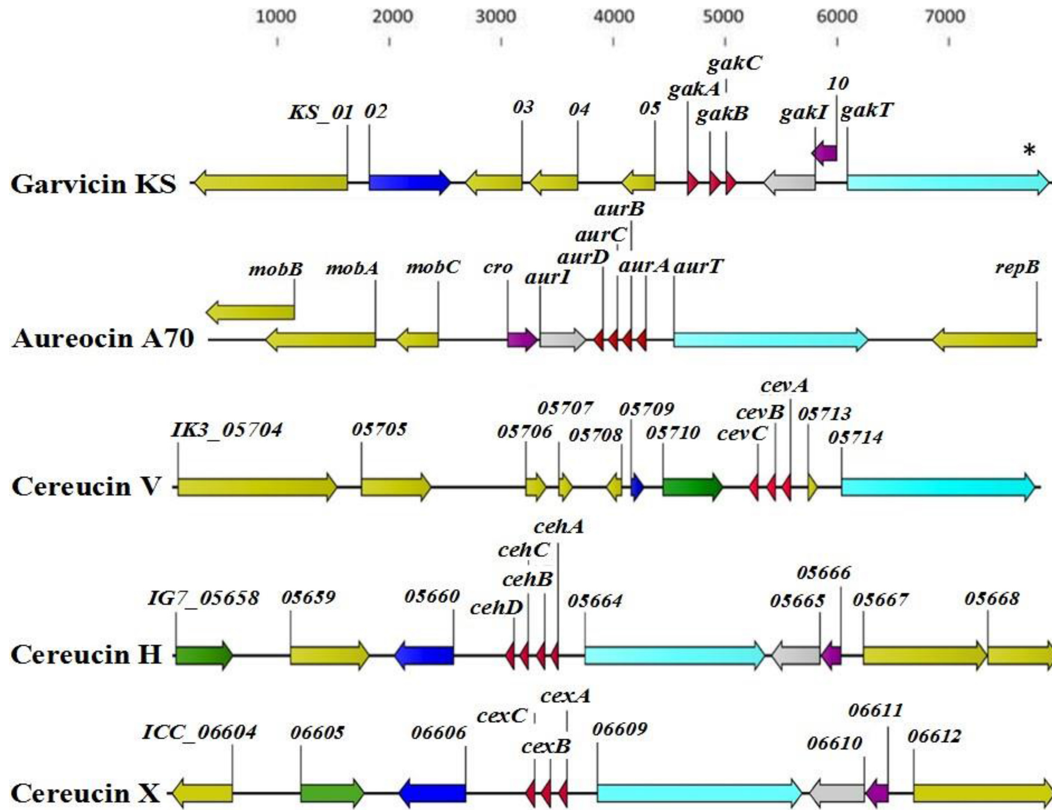

FIG 4 Bacteriocin loci of the multipeptide leaderless bacteriocin family. All loci contain bacteriocin structural genes (red), ABC transporter genes (light blue), and immunity or immunity-like genes (except for the cereucin V locus) (gray). Other conserved genes in the flanking regions are those homologous to the transposase gene (blue), mercury resistance protein gene (green), and Cro/cI protein gene (dark magenta). The remaining genes are homologous to genes for a DNA polymerase (KS_01), an acetyltransferase (KS_03), an AraC family transcriptional regulator (KS_04), and a DNA ligase (KS_05). Also shown are genes for mobilization-associated proteins B, A, and C (*mobB*, *-A*, and *-C*), replication protein Rep (*repB*), mobilization protein IK3_05704, hypothetical protein IK3_05705, replication protein IK3_05706, hypothetical protein IK3_05707, cold shock protein IK3_05708, hypothetical protein IK3_05713, hypothetical protein IG7_05659, RND transporter MFP subunit IG7_05667, macrolide ABC transporter IG7_05668, XRE family transcriptional regulator ICC_06604, and HyD family secretion protein ICC_06612. *, downstream of *gakT* were genes for two hypothetical peptides and a peptide transporter not related to the other proteins.

cochemical properties (most being small, cationic, and amphiphilic or hydrophobic) (34, 35).

The bacteriocin identity was confirmed by the use of synthetic peptides. The peptides individually exhibited no or very poor antimicrobial activity, but when the three peptides were combined in equimolar concentrations, they exhibited a potent activity, with MICs at nanomolar concentrations, confirming that the three peptides form a functional unit (Table 4).

```
ICC_06610   MERLKKWFSLNTHPDERIQQIEMKIWAQSGIIVLLIAFIDFIIRGAYLHRPFLEWAASLA
IG7_05665   MEKLKNWFSLSTHSDERIQQIEMKIWAQSGIVVLLLAFIDFIIRGLYLQRPFLEWAA-TL
aurI        -------------MDERQEIINKNMLIRSFGFLFILLIIYIAFLGVINVTITGHQLILIS
gakI        -MLYFGGKNMKKINDERIIKKDNEIITRTFILMFVLSLFYIVLFNKVVFFREQPQATIFS
                             ***    : ::  ::  .:::: :: : : .

ICC_06610   IIIFYMIFFFIKSILTGIYETDINNKEQLNEKLKEKMSNTLIFCFVAIGTTTYKYNLPED
IG7_05665   IIICYIVFFLIRSVLAGVYETDIHNKEQLNKKLKEKMVNTFIFCFVAISITTYRNQLPEN
aurI        IITLTSIYMWIDSFINKILYYDVQNNKEIKRKLSSCVTTLLVIDVAVLILAFLNKIDINI
gakI        IIIITTVYFIFDSFISKTLFVNIQEKNDVLKKVSHICSLIIAFDTLFILLSLTKKINIDL
            **    ::: : *.:      :::::::: .*:.      : :    :  :  .    :

ICC_06610   FIGWLSVIARFIILFAFLFGIQYLITKYTWYKNNKN
IG7_05665   TIGWLLVILKFIIVFSLIFGIQYLIAKFTWYKNNKN
aurI        SFLFLAILISFNIIVLSVY-YIILKFWLIWYK----
gakI        NLDTIIVLLSLNIFLFISY-YAILRLWVKWIK----
             :  : ::  : *..   :    *     * *
```

FIG 5 Clustal Omega alignment of garvicin KS family immunity proteins. Stars indicate identical amino acids, colons very similar amino acids, and periods similar amino acids.

We observed a 28-Da mass difference between the peptide masses obtained in MS and theoretical masses based on the DNA sequence, indicating formylation of the first methionine residues of the peptides, a feature that distinguishes leaderless bacteriocins from bacteriocins with leader sequences (36). Formylation generally blocks Edman degradation, but *N*-formyl groups are easily removed at low pH (36). Such acidic conditions were encountered during the purification due to the presence of TFA in the fast-performance liquid chromatography buffers. Thus, most of the purified GakA was likely spontaneously deformylated before Edman sequencing.

Interestingly, after the first RPC step of purification, two peaks with antimicrobial activity were detected (Fig. 1). MS of the first peak RPC fractions showed the presence of three peptides with masses of 3,114.6, 3,174.4, and 3,467.8 Da—all about 12 Da smaller than the masses of formylated GarKS peptides (Fig. 2). This mass difference could be due to oxidation (plus 16 Da) and deformylation (minus 28 Da) of the formylated methionine residues during purification or storage, resulting an oxidized methionine [Met(O)] with a mass 12 Da smaller than that of the formylated form. In fact, such modifications have been described for some other leaderless bacteriocins (36). The resulting oxidized peptides would be more hydrophilic. This notion is in line with the observation that these peptides were eluted with a lower concentration of 2-propanol (31%) than the peptides with formylated and nonoxidized methionine residues (34%).

A search for garvicin KS homologues in public sequence databases revealed several hits of significance, all from genome sequences of *Bacillus* and *Staphylococcus* species (Table 2). One of them is the known four-peptide bacteriocin AurA70. This is a plasmid-encoded, four-peptide (30- to 31-residue) bacteriocin produced by *S. aureus* A70 (24). The strain is involved in bovine mastitis and is quite common among *S. aureus* strains in Brazil and Argentina (37, 38).

The remaining three bacteriocins were found in different *B. cereus* genome sequences with some of their peptides annotated just as hypothetical proteins. Some of the peptides were even overlooked by automatic annotation software because of their relatively small sizes (26 to 30 residues).

All these bacteriocins have several features in common: they all are leaderless, each is made up by 3 or 4 small peptides, and their genetic organizations are quite similar, with all structural genes being organized in operon-like structures adjacent to genes homologous to those involved in immunity and transport. Moreover, neighboring the bacteriocin structural genes, there are other genes, which are conserved but apparently not involved in bacteriocin biosynthesis (Fig. 4). These include genes encoding Cro/cI family proteins and integrases/transposases, i.e., genes normally associated with genetic mobile elements. In case of *B. cereus* loci, the identity level between the genes was up to 93 to 100%. Whether this conserved genetic organization is coincident or may have a biological link (e.g., with bacteriocins serving as a toxin-antitoxin system) remains to be investigated.

As shown in Table 4, all bacteriocins have relatively broad inhibitory spectra, with garvicin KS being the most active in the group. Bacteriocins produced by *B. cereus* were slightly more active than GarKS only against *Bacillus* species, which is in line with the general characteristic of bacteriocins, namely, that they are most active against species closely related to the producers.

It has been shown that tryptophan residues in the C termini of bacteriocins are important for their activity (39, 40). As shown in Fig. 3, there are three conserved tryptophan residues in GakA homologous peptides (W23, W26, and W33 in GakA). In the case of GakA, we found W26 to be the most important, since its replacement with A26 led to total loss of activity of the individual peptide GakA as well as the whole bacteriocin GarKS. On the other hand, replacement of W23 and W33 with an alanine reduced the activity of the resulting individual peptides only by half. Whether W26 plays a crucial role in interacting with a hydrophobic environment in the receptor, with the other peptides to form a functional bacteriocin unit, or in other unknown functions remains unknown.

In this study, we have identified a novel group of bacteriocins which share several physicochemical and genetic properties. Most remarkably, they all are leaderless multipeptide bacteriocins whose peptides show significant amino acid sequence similarity to each other, not only within each bacteriocin unit but also across the different bacteriocin units, indicating that these bacteriocins probably share the same ancestor. This group presently contains five members: AurA70, GarKS, CerX, CerH, and CerV, of which only AurA70 has been reported before. AurA70 and CerH consist of four peptides, although the latter can also be viewed as a three-peptide bacteriocin because one of the peptides is dispensable. The remaining are three-peptide bacteriocins. Among these bacteriocins, garvicin KS appears to be the most interesting because it has very potent activity against many pathogenic Gram-positive bacteria (*Listeria*, *Enterococcus*, *Bacillus*, and *Staphylococcus*), representing a great potential for antimicrobial applications.

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