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Characterization of Garvicin ML, a Novel Circular Bacteriocin Produced by *Lactococcus garvieae* DCC43, Isolated from Mallard Ducks (*Anas platyrhynchos*)▽

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***Lactococcus garvieae* DCC43 produces a bacteriocin, garvicin ML (GarML), with a molecular mass of 6,004.2 Da. Data from *de novo* amino acid sequencing by tandem mass spectrometry and nucleotide sequencing by reverse genetics suggested that the bacteriocin is synthesized as a 63-amino-acid precursor with a 3-amino-acid leader peptide that is removed by cleavage. Subsequently, a covalent linkage between the N and C termini forms the mature version of this novel 60-amino-acid circular bacteriocin.**

Ribosomally synthesized antimicrobial peptides (AP) are produced by many organisms, including mammals, birds, insects, plants, and microorganisms. In bacteria, such peptides are termed bacteriocins (10, 33), and those of lactic acid bacteria attract considerable interest as food preservatives (5, 7, 14). Many AP are more active than conventional antibiotics against pathogenic and drug-resistant Gram-positive bacteria yet display no toxicity toward eukaryotic cells (35). AP may have applications in human and veterinary medicine in the treatment of local and systemic bacterial infections (24, 40, 42).

Bacteriocins have been classified into two major groups: class I lantibiotics with posttranslationally modified amino acids and class II nonlantibiotics with nonmodified amino acids (7, 34). Circular bacteriocins may constitute a new class (18, 23, 28, 29). The circular structure appears to enhance the thermodynamic stability and structural integrity of the peptide to improve its biological stability and activity (17). To date, a few circular bacteriocins are known: enterocin AS-48 (12), reuterricin 6 (43), acidocin B (27), butyrivibriocin AR10 (19), gassericin A (20), circularin A (23), subtilosin A (22), uberolysin (46), carnocyclin A (30), and lactocyclicin Q (41). These bacteriocins can be further classified according to their primary structures, biochemical characteristics, and genetic arrangements (21, 29). This study reports a novel circular bacteriocin, garvicin ML (GarML), produced by *Lactococcus garvieae* DCC43, isolated from Mallard ducks (*Anas platyrhynchos*) (39).

Strains and genetic techniques. Antimicrobial activity was evaluated by agar diffusion tests (ADT) and microtiter plate assays (MPA) as previously described (39) (Table 1). Plasmids were isolated using a midi kit (Qiagen) with added lysozyme (40 mg/ml) and mutanolysin (500 U/ml). Plasmid-Safe ATP-dependent DNase (Epicentre) eliminated residual genomic DNA. Genomic DNA, isolated as previously described (38), was digested with blunt-end-generating restriction enzymes, and fragments were ligated to an EcoRV-digested pCR-Blunt II-TOPO vector (Invitrogen). PCR was done in 50-μl mixtures, using 100 pmol of each primer and 1 U of Phusion high-fidelity DNA polymerase (Finnzymes). PCR fragments were isolated with QIAquick kits for purification or gel extraction (Qiagen). DNA was sequenced with a PRISM BigDye terminator cycle sequencing kit and an automatic DNA sequencer, model 377 (Applied Biosystems). Homology searches, using the BLAST algorithm (2), were done from the website of the National Center for Biotechnology Information (NCBI).

Purification of the bacteriocin produced by *L. garvieae* DCC43 and mass spectrometry analysis. The supernatant of an overnight culture of *L. garvieae* DCC43 was subjected to peptide purification by ion exchange chromatography on a HiPrep 16/10 SP-XL column (GE Healthcare Biosciences) and two cycles of reversed-phase chromatography on a reversed-phase Resource RPC column (GE Healthcare Biosciences) and a Sephasil peptide C_8 5-μm ST 4.6/100 column (Amersham Biosciences) integrated onto an Äkta purifier fast protein liquid chromatography system (FPLC). The molecular weight of the bacteriocin was determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) as described previously (9). Analysis of the purified entity, garvicin ML (GarML), showed that only the $[M + H]^+$ and $[M + 2H]^{2+}$ peaks of the bacteriocin were present, suggesting that the monoisotopic molecular mass of GarML is 6,004.2 Da (Fig. 1).

Proteolytic digestion of purified garvicin ML and *de novo* MS-MS peptide mapping. Initial efforts to determine the N-terminal amino acid sequence of GarML by Edman degradation failed, suggesting that the peptide was either cyclic or N-terminally blocked. However, although various peptide fragmentation procedures are available (4, 23, 41), GarML was digested by trypsin, either by a standard overnight protocol or

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TABLE 1. Antimicrobial activities and inhibitory spectrum of fractions generated from the purification of garvicin ML produced by *L. garvieae* DCC43[a]

Indicator strain	Source[b]	Halo of inhibition (mm^2) using indicated garvicin ML fraction[c]					
		SN	AS	GF	SE	OE	RP
Lactobacillus reuteri 20016	DSM	—[d]	—	—	51	369	378
Lactobacillus helveticus 15009	ATCC	—	—	22	26	129	492
Lactobacillus curvatus 2739	NCFB	—	—	—	—	221	177
Lactobacillus casei 334	ATCC	83	215	153	191	1,048	1,174
Lactobacillus acidophilus 4356	ATCC	—	—	—	25	149	236
Lactobacillus sakei 2714	NCFB	94	204	163	249	1,445	1,445
Lactococcus lactis BB24	FVM	97	186	150	183	941	928
Lactococcus lactis NZ9000	NIZO	—	69	37	77	163	163
Lactococcus lactis DPC5598	DPC	163	98	113	141	1,075	1,541
Lactococcus garvieae 5274	CECT	—	67	71	94	684	617
Lactococcus garvieae 5806	CECT	334	482	413	561	2,807	2,807
Lactococcus garvieae 5807	CECT	291	438	441	529	2,603	2,705
Lactococcus raffinolactis 988	CECT	—	—	—	10	228	228
Pediococcus acidilactici 347	FVM	94	193	153	235	1,398	1,398
Pediococcus pentosaceus FBB61	TNO	21	144	108	193	1,131	1,131
Enterococcus faecium P13	FVM	65	201	160	232	1,217	1,291
Enterococcus faecium L50	FVM	—	63	47	83	574	719
Enterococcus faecalis DBH18	FVM	—	—	—	—	25	25
Enterococcus faecalis P4	IFR	—	—	—	—	86	123
Propionibacterium sp. P6	NCDO	62	119	111	130	684	190
Propionibacterium acidipropionici 563	NCDO	75	153	105	132	662	864
Clostridium tyrobutyricum 3,5 CT	TNO	55	124	83	124	719	651
Clostridium tyrobutyricum 1754	NCDO	58	129	105	126	802	839
Clostridium perfringens 376	CECT	—	45	29	31	351	352
Clostridium botulinum 551	CECT	—	59	174	227	1,232	574
Listeria monocytogenes 4032	CECT	—	22	215	289	1,574	662
Listeria ivanovii 913	CECT	—	139	273	353	1,978	1,020
Listeria seeligeri 917	CECT	34	87	212	292	1,509	684
Listeria grayi 931	CECT	—	127	135	191	1,075	742
Listeria welshimeri 919	CECT	—	—	158	258	1,291	452
Brochothrix thermosphacta 847	CECT	—	—	—	31	255	283
Pseudomonas fluorescens 378	CECT	—	—	—	—	—	—
Escherichia coli JM109	Invitrogen	—	—	—	—	—	—
Escherichia coli MC1000	NIZO	—	—	—	—	—	—
Salmonella paratyphi 554	CECT	—	—	—	—	—	—
Salmonella Typhimurium 443	CECT	—	—	—	—	—	—
Salmonella enteritidis 4396	CECT	—	—	—	—	—	—
Streptococcus pneumoniae FQ26	HRC	69	156	100	110	599	590
Streptococcus pneumoniae 67620	HRC	65	135	94	128	707	596
Streptococcus pneumoniae 15 M-1047	HRC	58	128	96	139	790	790

[a] Purification of the bacteriocin was described by Sánchez et al. (39).

[b] Source abbreviations: ATCC, American Type Culture Collection (Rockville, MD); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); DPC, Teagasc Dairy Products Research Centre, Moorepark, Fermoy (County Cork, Ireland); DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); FVM, Facultad de Veterinaria de Madrid (Madrid, Spain); HRC, Departamento de Microbiología, Hospital Universitario Ramón y Cajal (Madrid, Spain); IFR, Institute of Food Research (Norwich, United Kingdom); NCDO, National Collection of Dairy Organisms (Reading, United Kingdom); NCFB, National Collection of Food Bacteria (Reading, United Kingdom); NIZO, Department of Biophysical Chemistry, NIZO Food Research (Ede, Netherlands); TNO, Nutrition and Food Research (Zeist, Netherlands).

[c] Antimicrobial activity was determined by the agar diffusion test (ADT), and the area of the halo of inhibition (mm^2) is shown. Fraction abbreviations: SN, supernatant; AS, ammonium sulfate precipitation; GF, gel filtration; SE, Sepharose fast flow eluate; OE, octyl Sepharose eluate; RP, reversed-phase eluate diluted 5-fold (vol/vol) with 30% 2-propanol containing 0.1% trifluoroacetic acid.

[d] —, no halo of inhibition.

in a micropipette tip (16, 37). To facilitate *de novo* tandem mass spectrometry (MS-MS) peptide mapping, the peptides were derivatized with a Lys tag and/or 4-sulfophenyl isothiocyanate (SPITC; Sigma-Aldrich) (26, 36). Digestion of GarML with trypsin produced two major peptide fragments of 1,652 Da and 3,581 Da, and their amino acid sequences are shown in Fig. 2A.

Identification of the structural gene and DNA and protein sequence analysis of garvicin ML. Based on the known amino acid sequence of the two major peptide fragments, four degenerate primers (DP7 to DP10) were designed for PCR amplification and DNA sequencing of the gene encoding mature GarML (Fig. 2A). Only the primer pair DP7/DP10 produced a PCR fragment (119 bp) that matched the amino acid sequence of the trypsin digests of GarML (Fig. 2B). New primers were designed by primer walking, and specific PCR fragments were sequenced and assembled into a 264-bp contig. As a result, the DNA sequence of the structural gene encoding GarML, termed *garML*, was obtained. The *garML* gene consisted of a 189-bp open reading frame (ORF) encoding a primary translation product of 63 amino acid residues, preceded by a putative ribosomal binding site (GGAGG) upstream of the methi-

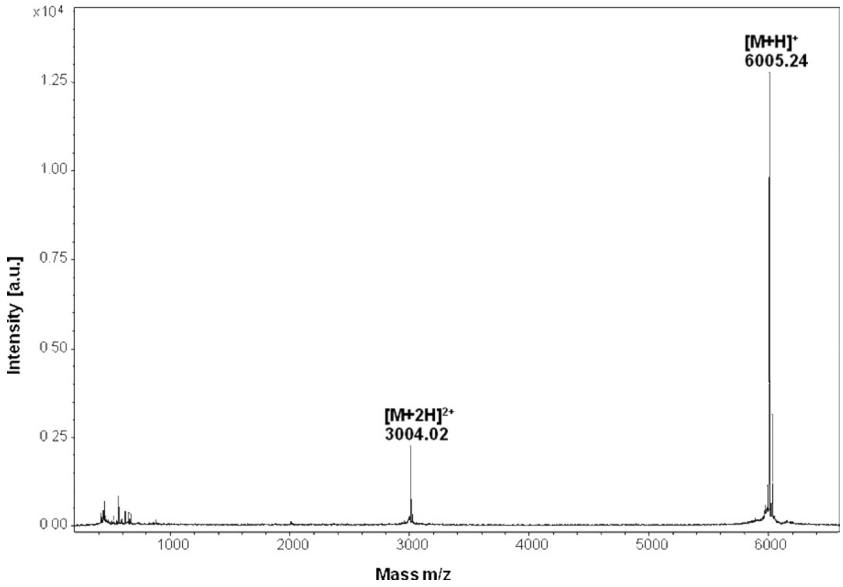

FIG. 1. Mass spectrometry analysis of the purified garvicin ML produced by *L. garvieae* DCC43. a.u., absorbance units.

onine translation initiation codon (Fig. 2C). The deduced amino acid sequence of the minor trypsin digest from GarML (IAALVATGMAAGVAK) permitted the determination of the exact point of circularization, suggesting that GarML is synthesized as a 63-amino-acid precursor peptide which is processed between Asp^3 and Leu^4 to produce the 60-amino-acid mature peptide (Fig. 2). We postulate that the putative leader peptide (tripeptide) of the GarML precursor is cleaved off and cyclization takes place between the N-terminal Leu^4 and the C-terminal Ala^{63} by a peptide bond (Fig. 3). *garML* is also carried on a plasmid (results not shown). To date, only one other *L. garvieae* bacteriocin, garviecin L1-5, produced by *L. garvieae* L1-5, isolated from raw cow's milk, has been reported (45).

(A)

```
Fragment  : I/L  A  A  I/L  V  A  T  G  M  A  A  G  V  A  K  (1,652 Da)
Primer DP7:                   5´-ACNGGNATGGCNGCNGGNGTNGC-3´
Primer DP8:             3´-CANCGNTGNCCNTACCGNCGNCC-5´

Fragment  : D  I/L  A  T  A  I/L  S  I/L  F  S  G  A  F  T  A  A  G  G I/L M  A  I/L  I/L  K (3,581 Da)
Primer DP9:                                  5´-GGNGCNTTYACNGCNGCNGGNGG-3´
Primer DP10:                                        3´-TGNCGNCGNCCNCCNDANTACCG-5´
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(B)

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  1  ACTGGGATGGCTGCTGGTGTTGCAAAAACTATTGTTAATGCCGTTAGTGCTGGTATGGAT
       T  G  M  A  A  G  V  A  K  T  I  V  N  A  V  S  A  G  M  D
 61  ATTGCCACTGCTTTATCATTGTTCTCAGGAGCTTTTACTGCAGCAGGCGGAATAATGGC
       I  A  T  A  L  S  L  F  S  G  A  F  T  A  A  G  G  I  M  A
```

(C)

```
  1  TAAAATAAAGTAATTTATAGGAGGATTATTATGTTTGATTTAGTCGCGACTGGAATGGCT
                        RBS         M  F  D ↑ L  V  A  T  G  M  A
 61  GCAGGTGTAGCAAAAACTATTGTTAATGCCGTTAGTGCTGGTATGGATATTGCCACTGCT
       A  G  V  A  K  T  I  V  N  A  V  S  A  G  M  D  I  A  T  A
121  TTATCATTGTTCTCAGGAGCTTTTACTGCAGCTGGGGGAATTATGGCACTCATTAAAAAA
       L  S  L  F  S  G  A  F  T  A  A  G  G  I  M  A  L  I  K  K
181  TATGCTCAAAAGAAATTATGGAAACAGCTTATTGCTGCATAACTTCTGGAAAAATCAATA
       Y  A  Q  K  K  L  W  K  Q  L  I  A  A  *
241  AAAAATAAAAGTAACTTATATAAG
```

FIG. 2. Determination of the amino acid and nucleotide sequences of garvicin ML produced by *L. garvieae* DC443. (A) Amino acid sequences obtained by *de novo* MS-MS peptide mapping of the major peptide fragments obtained after trypsin digestion of garvicin ML, and the degenerate primers designed based on the sequences. (B) The 119-bp nucleotide sequence obtained after amplification of genomic DNA from *L. garvieae* DCC43 with primers DP7/DP10, and its deduced amino acid sequence. The nucleotide sequences corresponding to primers DP7 and DP10 are underlined. (C) From the above-cited nucleotide sequence, using reverse genetics, a sequence of 264 bp and its deduced amino acid sequence were obtained. A putative ribosome binding site (RBS) is underlined. An asterisk identifies the translation stop codon. The predicted cleavage site of the leader peptide is indicated by a vertical arrow.

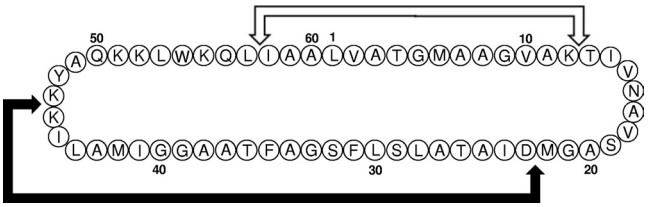

FIG. 3. Circular structure of garvicin ML. Double-ended arrows indicate locations of the major peptide fragments obtained after trypsin digestion of garvicin ML. The white arrow defines the 1,652-Da fragment, and the black arrow the 3,581-Da fragment.

GarML shares limited amino acid similarity (30% identity) with carnocyclin A (CclA), a circular bacteriocin from *Carnobacterium maltaromaticum* UAL307 (30), and a lower similarity (28% identity) with enterocin AS-48, produced by *Enterococcus faecalis* S-48 (12). The predicted secondary structure of GarML, obtained with both the Jpred3 (6) and the PSIPRED (32) protein structure prediction server, as well as by modeling the three-dimensional (3-D) structure of GarML with DeepView and SWISS-MODEL (http://spdbv.vital-it.ch) and ESyPred3D (25), suggests that GarML folds into a compact globular bundle comprised of four conserved α-helices enclosing a compact hydrophobic core. The structures of CclA, enterocin AS-48, circularin A, uberolysin, and lactocyclicin Q, as well as the predicted model for GarML, show that a cluster of basic amino acid residues, such as the Lys^{46}, Lys^{47}, Lys^{52}, Lys^{53}, and Lys^{56} residues for GarML (Fig. 3), impart a highly localized positive charge on the surface of the peptide (31). These conserved residues are likely responsible for attracting the peptides to the surface of the negatively charged membrane. Differences in antimicrobial activities among the circular bacteriocins may result from variations in the surface features of the conserved framework (17, 31). The 3-amino-acid-long leader peptide (MFD) of the GarML precursor is one of the shortest described for circular bacteriocins (23, 30, 41). However, the function of the leader peptides in the targeting and translocation of circular bacteriocins and how cyclization from the linear precursors occurs are still not understood (8, 29, 30).

Sensitivity of the bacteriocin to heat, pH, and proteolytic enzymes and antimicrobial spectrum of garvicin ML. GarML showed resistance to temperature (80 and 100°C) and alkaline and acid pH (2 to 10), and to digestion by trypsin, pepsin, papain, and proteinase K (results not shown). The resistance of GarML to proteolytic enzymes is not due to the absence of digestion sites but to the inaccessibility of the recognition sites, probably due to a tightly folded three-dimensional structure. This could make the bacteriocin less susceptible to digestion by endoproteinases and increase its spectrum of activity (23). GarML shows a higher antibacterial activity and a broader antimicrobial spectrum as it is increasingly purified (Table 1), probably due to removal of antimicrobial inhibitors, disaggregation of the bacteriocin, or changes in conformation of the bacteriocin in the hydrophobic solvent. However, different from other circular bacteriocins (13, 41), no activity against any Gram-negative bacteria was recorded, suggesting that its mode of action may be different. Nevertheless, GarML inhibits other *L. garvieae* strains, and this is an interesting observation. *L. garvieae* is the etiological agent of lactococcosis, an emergent hyperacute, hemorrhagic septicemia that affects a range of fish and crustacea worldwide and has a considerable sanitary and economic impact in the freshwater and marine fish-farming industry (1, 11, 44). Several strategies, mostly based on the use of vaccines, bacteriocins, and probiotics, are being developed to combat *L. garvieae* in fish farming (3, 15). Further efforts are needed to determine the role of *L. garvieae* DCC43 as producer of a highly active circular bacteriocin against spoilage and pathogenic bacteria or as a potential probiotic against infections caused by other *L. garvieae* strains.

Nucleotide sequence accession number. The nucleotide sequence of the structural gene encoding garvicin ML has been deposited in the GenBank database under accession number GU205098.

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