

Sakacin G, a New Type of Antilisterial Bacteriocin

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Received 23 April 2002/Accepted 2 September 2002

Sakacin G is a 37-amino-acid-residue-long class IIa bacteriocin produced by *Lactobacillus sake* 2512, which is encoded by the duplicated structural genes *skgA1* and *skgA2*. Sakacin G appears to be unique and seems to be an intermediate between pediocin-like bacteriocins, according to its double-disulfide bridges required for antimicrobial activity, and mesentericin-like bacteriocins in terms of sequence homologies, inhibition spectrum, and specific activity.

Many of the lactic acid bacteria are capable of inhibiting growth of some gram-positive bacteria, such as pathogenic species like *Listeria monocytogenes*, by secreting antimicrobial compounds, including peptides and proteins, also called bacteriocins. Among antibacterial peptides, lantibiotics, characterized in their primary structure by the presence of modified amino acid residues, have been widely studied and are used in many countries as preservatives in food products (29). Another group of peptides that do not bear any modified amino acid residue form a subclass called antilisterial bacteriocins, also known as class IIa bacteriocins (11) or cystibiotics (18). These peptides share strong structural homologies in their N-terminal domain (Fig. 1) with the presence of one disulfide bond. Their C-terminal domain is highly more variable. However, some of these bacteriocins, such as pediocin PA-1 (23), enterocin A (3), coagulin (22), and divercin V41 (24), are characterized by the presence of a second disulfide bond in the C-terminal region (Fig. 1). Moreover, in a previous work (14), we showed that these double-disulfide-bond bacteriocins exhibited higher specific activity than those with only two cysteine residues: i.e., mesentericin Y105 (13), sakacin A (2), and sakacin P (33).

A new method for detection of antilisterial bacterial strains was recently developed and applied to select several strains from the Rhodia Food collection that produce bacteriocins (31). One strain, *Lactobacillus sake* 2512, appeared to produce an unknown bacteriocin. In the present paper, we report on the purification and biochemical characterization of a new and structurally original class IIa bacteriocin, sakacin G. Characterization of part of the genetic elements required for the production of this antimicrobial peptide is also described. Peculiar features in the proteic and nucleic structures found are discussed.

Bacteriocin characterization and purification. Antilisterial activity of *Lactobacillus sake* 2512 was detected by a new luminescence-based method (31). Briefly, luminescent target strains (*Listeria* and *Enterococcus*) were obtained by cloning bacterial luciferase genes. When these strains were exposed to

a culture supernatant containing antilisterial activity, their light emission was rapidly suppressed, allowing rapid detection of bacteriocins. The bacteriocin purification was conducted by a three-step procedure developed previously (14). The fraction of the major 23-min peak, collected during the reverse-phase high-performance liquid chromatography final purification step, was active against *Listeria ivanovi* BUG 496. The molecule, analyzed by electrospray ionization-mass spectrometry as previously described (14), showed a molecular mass of $3,834.32 \pm 0.31$ Da. Database screening revealed that the molecular mass of none of the previously described bacteriocins from lactic acid bacteria corresponds to this molecular mass. The amount of sakacin G purified from 100 ml of culture supernatant was estimated by protein titration with the bicinchoninic acid protein assay kit (Sigma) to be 120 μg and corresponds to a yield of 55% of recovered activity, measured with the critical dilution assay previously described (13).

Specific activity of sakacin G toward *L. ivanovi* BUG 496 was determined by the method used previously for other class IIa bacteriocins (14). The MIC of the new bacteriocin was 50.10^{-3} $\mu\text{g/ml}$, comparable to the MICs of mesentericin Y105, and sakacins A and P (33.10^{-3} , 65.10^{-3} , and 65.10^{-3} $\mu\text{g/ml}$, respectively) and significantly higher than those of the double-disulfide-bond-containing bacteriocins pediocin PA-1, enterocin A, and divercin V41 (4.10^{-3} , 10.10^{-3} , and $1.4.10^{-3}$ $\mu\text{g/ml}$, respectively).

Finally, the activity of the sakacin G toward 19 bacterial strains was tested by the well diffusion method, and the results are shown in Table 1. The inhibition spectrum of this bacteriocin appeared to be narrow and limited to the *L. sake* and *Pediococcus cerevisiae* strains tested, as far as lactic acid bacteria are concerned. This peptide, like the class IIa bacteriocins, appeared to be active toward all *Listeria* and *Enterococcus faecalis* strains tested. Compared to other class IIa bacteriocins tested against the same target strains (14), sakacin G is not active toward *Lactobacillus casei* DSM 20011 and *Enterococcus faecium* ENSAIA 631, as demonstrated for mesentericin-like peptides (mesentericin Y105, sakacin A, and sakacin P) and contrary to pediocin-like bacteriocins (pediocin PA-1, enterocin A, and divercin V41).

Structural characterization. The first 34 amino acid residues of the N-terminal sequence of sakacin G were determined by Edman degradation ("Centre Commun d'Analyses du CNRS,"

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Bacteriocin	1	10	20	30	40	Reference
Sakacin G	KYYGNGVSCNSHGC	SVNWGQAWTCGVNHLANGGHGVC				This work
Mesentericin Y105	KYYGNGVHCTKSGCS	VNWGEAASAGIHLRANGGNFW				(13)
Leucocin A	KYYGNGVHCTKSGCS	VNWGEAFSAGVHRLANGGNFW				(15)
Leucocin C	KNYGNGVHCTKKGCS	VDWGYAATNIANNVSNGLTG				(27)
Sakacin P	KYYGNGVHCGKHSC	TVDWGTAFIGNI GNNAANWATGWNAGG				(33)
Mundticin	KYYGNGVSCNKKGCS	VDWGKAIGIIGNNSAANLATGGAAGWSK				(4)
Sakacin A	ARSYNGVVCNNKCC	VNRGEATQSIIGGMSGWASGLAGM				(2)
Piscicocin V1a	KYYGNGVSCNKNK	CTVDWSKAIGIIGNNAANLTGGAAGWNKG				(6)
Bacteriocin BM1	AISYNGVVCNKEKC	WVNKAENKQAITGIVIGGWASSLAGMGH				(28)
Bacteriocin B2	VNYGNGVSCSKTKCS	VNWGQAFQERYTAGINSFVSGVASGAGSIGRRP				(28)
Bavaricin MN	TKYYGNGVVCNSK	KCVWDWGOAAGGIGQTVVxGWLGGAI PGK				(20)
Enterocin P	ATRSYNGVVCNNSK	CVWNWGEAKENIAGIVISGWASGLAGMGH				(8)
Bacteriocin 31	ATYYGNGLYCNKQ	KCVWDWNKASREIGKIVNGWVQHGFWAPR				(34)
Bifidocin B	KYYGNGVTCGLHDC	RVRDRGKATCGIINNGGMWGDIG				(35)
Pediocin PA-1	KYYGNGVTCGKHSC	SVDWGKATTCIINNGAMAWATGGHQGNHCK				(23)
Coaguln	KYYGNGVTCGKHSC	SVDWGKATTCIINNGAMAWATGGHQGTHCK				(22)
Enterocin A	TTHSGKYYGNGV	CTKNKCTVDWAKATTCIAGMSIGGFLGGAI PGKC				(3)
Divercin V41	TKYYGNGVVCNSK	KCVWDWGOASGIGQTVVGGWLGGAI PGKC				(24)
Consensus	<u>YYGNGV</u>	<u>C</u>	<u>C</u>	<u>V</u>	<u>WG</u>	<u>A</u> <u>I</u>

FIG. 1. Sequence alignment of class IIa bacteriocins. Leucocin A is identical to leucocin B (12). Piscicocin V1a is identical to piscicocin 126 (19), sakacin A is identical to curvacin A (32), carnobacteriocin BM1 is identical to piscicocin V1b (6), and pediocin PA-1 is identical to pediocin Ach (26) and pediocin SJ-1 (30). Bavaricin A (21) is probably identical to sakacin P. Residues are numbered according to the sequence of sakacin G. The consensus sequence includes residues conserved by at least 75%. The residues conserved by more than 90% are underlined.

IBCP, Lyon, France). The sequence KYYGNGVXSNSHG XSVNWGQAWTXGVNHLANGGH was obtained.

The class IIa consensus sequence YGNGV showed that sakacin G belongs, as expected according to its inhibition spectrum, to class IIa bacteriocins (Fig. 1). Moreover, the three undetermined residues at positions 9, 14, and 24 could be attributed to three cysteines by homology with other class IIa bacteriocin sequences (Fig. 1). However, the calculated molecular mass of this 34-residue peptide with three cysteines was lower than the measured molecular weight of 3,834.32, indi-

cating that this sequence was incomplete and convincing us to clone and sequence the sakacin G structural gene.

Cloning and sequencing. The sequence of the locus involved in bacteriocin expression was analyzed by a reverse genetic strategy. The bacterial strains, genetic constructs, and oligonucleotide sequences used in this study are described in Table 2. First, the structural bacteriocin gene was amplified by PCR with two degenerated primers, SakG01 and SakG02, based on the N-terminal amino acid sequence of sakacin G. The amplification led to a 100-bp DNA fragment, which was cloned into the pGEM-T cloning vector, leading to pJMBYC01. The 560-bp *Pvu*II fragment from pJMBYC01, including the cloned fragment, was used as a hybridization probe in Southern blot experiments to locate the structural gene on the *L. sake*2512

TABLE 1. Inhibition spectrum of culture supernatant of *L. sake* 2512 containing sakacin G

Indicator strain	Inhibition zone diam (mm) ^a
<i>Lactococcus lactis</i> ATCC 11454	0
<i>Leuconostoc paramesenteroides</i> DSM 20288	0
<i>Leuconostoc mesenteroides</i> DSM 20484	0
<i>Leuconostoc mesenteroides</i> DSM 20240	0
<i>Lactobacillus delbrueckii</i> DSM 20081	0
<i>Lactobacillus plantarum</i> DSM 20174	0
<i>Lactobacillus brevis</i> DSM 20054	0
<i>Lactobacillus casei</i> DSM 20011	0
<i>Lactobacillus sake</i> 2515	1
<i>Pediococcus acidilactici</i> ENSAIA 583	0
<i>Pediococcus cerevisiae</i> IP 5492	1
<i>Enterococcus faecium</i> ENSAIA 631	0
<i>Enterococcus faecalis</i> IP 5430	2
<i>Enterococcus faecalis</i> ENSAIA 636	1
<i>Enterococcus durans</i> ENSAIA 630	2
<i>Listeria innocua</i> 8811	3
<i>Listeria monocytogenes</i> EGDe	2
<i>Listeria monocytogenes</i> LO28	2
<i>Listeria ivanovi</i> BUG 496	6

^a Fifty microliters of a 550-AU/ml active culture supernatant was tested.

TABLE 2. Plasmids and oligonucleotides used in this study

Plasmid or oligonucleotide	Relevant characteristics ^a	Source or reference
Plasmids		
pGEM-T	Ap ^r , PCR product cloning vector, 3 kb	Promega
pZErO-2	Kan ^r , cloning vector, 3.3 kb	Invitrogen
pJMBYC01	pGEM-T::100-bp <i>skgA1</i> PCR product, 3.1 kb	This work
pJMBYC02	pZErO-2::2.1-kb <i>Hind</i> III fragment of a plasmid from <i>L. sake</i> 2512, 5.4 kb	This work
Oligonucleotides		
SakG01	5' AAR TAT TAT GGN AAY GGN GT 3'	
SakG02	5' ACA TGA TGN CCN CCR TTN GC 3'	

^a Ap^r, ampicillin resistant; Kan^r, kanamycin resistant.

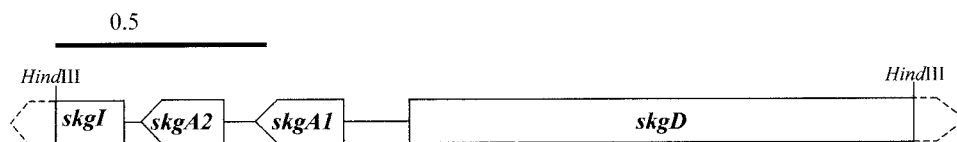


FIG. 2. Schematic representation of the sequenced 2.1-kbp *HindIII* fragment isolated from plasmid pJMB35. A size bar (0.5 kbp) is shown in the upper left corner.

DNA (data not shown). From the plasmid preparation of *L. sake*2512, the probe revealed that a bacteriocin gene was carried by a plasmid with an approximate size of 35 kbp, named pJMB35. After restriction, the probe hybridized to a 2-kbp *HindIII* fragment and to a 9-kbp *EcoRI* fragment. The *HindIII* fragment was purified and cloned into the pZERO2 plasmid, generating pJMBYC02.

The 2,047-bp cloned sequence was determined and analyzed. Analysis revealed two complete open reading frames (ORFs), *skgA1* and *skgA2*, in addition to two truncated ORFs, *skgI* and *skgD* located on both sides of the cloned fragment (Fig. 2). The presumed *skgA1*, *skgA2*, and *skgI* genes were in the opposite orientation to *skgD*. Each ORF is preceded by a potential ribosome binding site (RBS).

Pre-sakacin G coding genes. The *skgA1* and *skgA2* genes both code for a 55-amino-acid protein sharing an identical sequence (residues 19 to 55) (Fig. 3).

Positions 1 to 18 in the sequences of the SkgA1 and SkgA2 proteins (Fig. 3) differed only by 3 residues and showed strong homologies with the sequences of class IIa bacteriocin peptide leaders, which are involved in peptide transport by specific ABC transporters (11). More particularly, the GG terminal unit (17, 18) is characteristic of these leader sequences and is the maturation site of these bacteriocins.

The sequence from positions 19 to 52 corresponds exactly to the amino acid sequence obtained by Edman degradation mentioned above. The hypothetical presence of three cysteines at positions 9, 14, and 24 was confirmed. Moreover, three additional residues of glycine, valine, and cysteine, respectively, appeared to complete the protein sequence at its C terminus. Thus, the difference of 4 Da between the calculated molecular mass of this peptide (3,838.20 Da) and the measured molecular mass (3,834.32 Da) first indicated the presence of two disulfide bonds within sakacin G, as was already described for other antilisterial bacteriocins and second confirmed the presence of the three additional amino acids that were not initially detected.

This sakacin G locus exhibits a specific feature, namely, the

duplication of the sakacin G gene. This is the first example of a bacteriocin gene “duo” in the same operon. The mature sakacin G protein sequences deduced from the *skgA1* and *skgA2* genes are identical, whereas the leader sequences differ in three residues. Comparison of the nucleotide sequences of the *skgA1* and *skgA2* genes showed 95% homologies for mature bacteriocin sequences, whereas the leader sequence genes were only 78% identical. This indicates that conservation of the mature peptide sequence was more important than that of the leader and/or that the three amino acid positions modified in the leader are poorly involved in sakacin G maturation and secretion. Moreover, experiments (e.g., alternative *skgA1* and *skgA2* gene disruptions and/or fusion with two different reporter genes) must be conducted to determine whether both sakacin G structural genes are functional.

Role of disulfide bonds. The role of disulfide bonds in antilisterial activity of class IIa bacteriocins was studied in various peptides (11). Particularly, in comparative studies (10, 14), it has been demonstrated that bacteriocins with two disulfide bonds are more efficient antimicrobial agents and have broader spectra of activity than those that possess a single disulfide bridge. Surprisingly, despite two disulfide bonds, sakacin G displays a low specific activity and a quite narrow spectrum of activity, close to that of mesentericin-like bacteriocins. However, it has been shown that the disulfide bond reduction dramatically reduced the activities of pediocin-like bacteriocins (5, 7, 10, 25), whereas reduction treatments of mesentericin-like bacteriocins (10, 15, 16, 28) resulted in only a moderate decrease in activity. According to the ambiguous status of sakacin G, reduction experiments with dithiothreitol (12.5 mg of dithiothreitol per ml added to a 0.5-mg/ml bacteriocin solution) were conducted with sakacin G, pediocin PA-1, and mesentericin Y105 (data not shown). The results showed, as expected, that disulfide bond reduction had no effect on mesentericin Y105 activity, even though the same treatment induced a dramatic loss (about 10 times) of antilisterial activity for pediocin PA-1 and, interestingly, for sakacin G.

Moreover, sequence comparison between sakacin G and

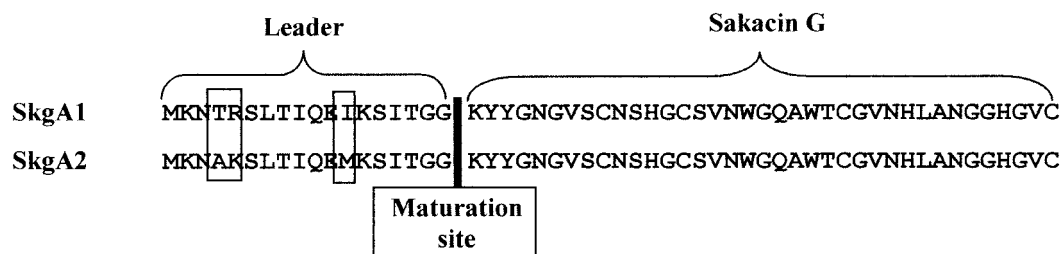


FIG. 3. Sequence alignments and relevant features from the proteic sequences deduced from the *skgA1* and *skgA2* genes. Differences between the two sequences are framed.

other class IIa bacteriocins showed better identities with mesentericin-like bacteriocins—i.e., leucocin A (65%) and mesentericin Y105 (62%)—than with pediocin-like bacteriocins, such as pediocin PA-1 (52%).

Consequently, we propose that sakacin G belongs to a new subgroup of class IIa bacteriocins with two disulfide bonds, one of them (C terminal) required for antibacterial activity like that of pediocin-like peptides, but with primary sequences, sizes, homologies, and specific activities closer to those of mesentericin-like bacteriocins.

Sequence analysis of other genes. The incomplete ORF *skgI* (Fig. 2) encodes a 54-residue protein. Comparison of this sequence with those in databases showed good homologies of the gene coding for SkgI with those coding for LccI (47%) and MesI (46%), which are the immunity proteins of leucocin A (15) and mesentericin Y105 (13), respectively. We can thus suggest that *skgI* encodes a sakacin G immunity protein.

The last incomplete gene, *skgD* (Fig. 2), encodes a 394-amino-acid protein. The position of the putative RBS points toward a TTG start codon, instead of a classical ATG. A search in protein databases revealed that SkgD shows great homologies with the following proteins of the ABC transporter family involved in bacteriocin secretion: pediocin PA-1, PedD and PapD (23, 26); sakacin P, SppT (17); sakacin A, SapT (1); and mesentericin Y105, MesD (13). The strongest homology was found with PlnG, potentially involved in expression of plantaricin A, a non-antilisterial class II bacteriocin (9). Thus, *skgD* may encode an ABC transporter specific for sakacin G.

New experiments to sequence the region of pJMB35 flanking the 2.1-kbp *Hind*III fragment would allow us to determine the complete sequences of *skgI* and *skgD*. This analysis could also show a downstream gene coding for SkgD, an accessory factor that is generally present near the ABC transporter-coding gene (11), with the exception of the gene coding for divercin V41, which contains a locus apparently devoid of the accessory factor gene (24). Subsequent cloning experiments with such operons in nonproducer strains would confirm that SkgI is the immunity protein to sakacin G and that SkgD is an ABC transporter dedicated to the secretion of sakacin G.

Nucleotide sequence accession number. The 2,047-bp cloned sequence of sakacin G was determined and analyzed. This nucleotide sequence, corresponding to partial *skg* locus, has been deposited in the GenBank database under accession no. AF395533.

We thank Véronique Laffitte from Rhodia Food for providing bacterial strains, Anne Venisse for critical review of the manuscript, and George Hette for technical assistance.

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