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NOTES

Variacin, a New Lanthionine-Containing Bacteriocin Produced by *Micrococcus varians*: Comparison to Lacticin 481 of *Lactococcus lactis*

D. PRIDMORE,[1] N. REKHIF,[1] A.-C. PITTET,[1] B. SURI,[2] AND B. MOLLET[1]*

Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, 1000 Lausanne 26,[1] and Animal Health Division, Ciba-Geigy Ltd., 4000 Basel,[2] Switzerland

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A new lanthionine-containing bacteriocin, variacin, displaying a broad host range of inhibition against gram-positive food spoilage bacteria, has been identified from two strains of *Micrococcus varians* isolated from meat fermentations. The new bacteriocin was purified, and its amino-terminal end and total amino acid composition were determined. The structural gene was isolated and analyzed. Variacin is resistant to heat and pH conditions from 2 to 10. Its primary sequence shows significant homology to lacticin 481 of *Lactococcus lactis*, which is more pronounced for the probacteriocin than for the leader sequence. Variacin, like lacticin 481, contains lanthionine and β-methyllanthionine residues, but its leader sequence clearly resembles nonlantibiotic leader sequences. In particular, the prepeptide contains glycine residues at positions −1 and −2 of the processing site.

The best-documented lanthionine-containing bacteriocin, nisin, was initially identified in 1933 as an antimicrobial agent produced by some natural *Lactococcus lactis* starter strains used in cheese fermentation (11). Today, nisin is accepted as a food additive by the Food and Drug Administration and mainly used to limit *Clostridium* spore outgrowth and toxin production in processed cheese and cheese spreads. However, the use of nisin and other lanthionine-containing bacteriocins in the food industry is rapidly expanding into new areas. In this work we describe the identification, purification, and characterization of a new lanthionine-containing bacteriocin from two strains of *Micrococcus varians*. The two bacterial strains are not identical and have different plasmid profiles (data not shown). They were both isolated from laboratory production trials of Italian-type raw salami fermentations and were part of the natural meat flora (5).

The bacteriocin activity produced by *M. varians* MCV8 and MCV12, variacin 8 and variacin 12, was determined by the agar well diffusion method (10). Bacteriocin activities were estimated by measuring the zones of inhibition ($diameter_{halo}$ minus $diameter_{well}$, in millimeters). Indicator bacteria were selected strains of lactic acid and food spoilage bacteria. The results showed that variacins 8 and 12 exhibit the same wide spectrum of activity (Table 1). Indeed, they inhibited all of the lactic acid bacteria tested, as well as gram-positive pathogenic and spoilage bacteria such as listeriae, staphylococci, and the vegetative cells and spores of clostridia and bacilli. However, no gram-negative bacteria were inhibited by variacin, a feature common to most known bacteriocins produced by gram-positive bacteria.

XAD-7 concentrates of variacin were obtained by incubating cell-free supernatants of 1-liter *M. varians* cultures with 20 g of Amberlite XAD-7 (Sigma, Buchs, Switzerland) for 1 h at 4°C, collecting the resin by filtration, and washing it twice with 1% sodium citrate. The XAD-7 was then packed into a column, and the antibacterial activity was eluted with 50% acetonitrile–0.1% trifluoroacetic acid (Sigma). The eluents were dried under vacuum and then resuspended in 50 ml of 50 mM phosphate buffer (pH 6.8) per 1-liter initial cell culture. Aliquots of such XAD-7 semipurified concentrates of variacins 8 and 12 were subjected to different heat and pH treatments. Samples were heated to 100°C for 15, 30, and 45 min, and the remaining inhibitory activities were assayed. The control sample was kept at room temperature. The results showed no change in the inhibitory activity of either variacin 8 or variacin 12. Other samples were adjusted to pH 4 and 7, heated to 115°C for 20 min, and tested for inhibitory activity. Again, the full antimicrobial activity was retained. Alternatively, samples were adjusted to pH 2, 4, 6, 8, or 10 with either 2 M NaOH or 2 M HCl and incubated at 37°C for 1 h. After the samples were readjusted to pH 7, the inhibitory activity was assayed and revealed no loss in comparison to the control sample, which was incubated at pH 7. Hence, the antimicrobial activity of variacin is resistant to heat and pH conditions from 2 to 10.

We investigated the biochemical nature of variacin by using the activities of several enzymes. Samples of XAD-7 concentrate were adjusted to pH 7.0 and incubated with 1 and 5 mg each of catalase (Sigma), pronase E (Sigma), proteinase K (Merck, Darmstadt, Germany), and ficin (Sigma) per ml for 1 h at 37°C. The control sample consisted of the same bacteriocin concentrate incubated at 37°C without enzyme. The activity of variacin was fully retained after incubation with catalase, which excludes H_2O_2 as being responsible for the antimicrobial activity. On the other hand, the results after incubation with different proteinases, which all completely inactivated the antimicrobial activity of the samples, indicate that protein is at least a major component of the active bacteriocin.

To purify the bacteriocin, 400 ml of an XAD-7 concentrate was loaded onto a 5-ml S-Resource fast protein liquid chro-

* Corresponding author. Phone: 41 21 785 8930. Fax: 41 21 785 8925.

TABLE 1. Spectrum of activity of variacin

Target bacteria	No. of strains tested	Inhibition zone[a]	
		S[b]	XAD-7[c]
Gram-positive bacteria			
Lactobacillus helveticus	5	+++	++++
Lactobacillus bulgaricus	4	+++	++++
Lactobacillus lactis	2	++	+++
Lactobacillus delbrueckii	2	+++	++++
Lactobacillus acidophilus	4	++	+++
Lactobacillus plantarum	2	+	++
Lactobacillus sake (LSK)	1	++	+++
Lactobacillus curvatus	1	++	+++
Leuconostoc mesenteroides	2	+	++
Streptococcus thermophilus	6	++	+++
Lactococcus lactis (SL2)	1	++	+++
Micrococcus varians (MCV8)	1	−	−
Micrococcus varians (MCV12)	1	−	−
Enterococcus faecalis	1	++	+++
Enterococcus faecium	2	++	++
Listeria innocua	1	++	+++
Listeria monocytogenes	1	++++	++++
Listeria welhia	1	++	+++
Clostridium botulinum	4	ND[d]	+++
Clostridium sp.	6	ND	++
Staphylococcus aureus	4	ND	++
Staphylococcus carnosus	2	ND	+++
Staphylococcus saprophyticus	2	ND	++
Staphylococcus sp.	4	ND	+
Bacillus subtilis	6	ND	++
Bacillus cereus	5	ND	++
Bacillus pumilis	2	ND	+++
Gram-negative bacteria			
Escherichia coli	1	−	−
Enterobacter cloacae	1	−	−
Salmonella thyphimurium	1	−	−
Salmonella anatum	1	−	−
Pseudomonas fluorescens	1	−	−

[a] −, no zone of inhibition; +, zone of inhibition between 1 and 5 mm in diameter; ++, zone of inhibition between 6 and 9 mm in diameter; +++, zone of inhibition between 10 and 18 mm in diameter; ++++, zone of inhibition between 19 and 28 mm in diameter.
[b] Inhibitory activity tested with supernatant adjusted to pH 7.
[c] Inhibitory activity tested with supernatant concentrated on XAD-7.
[d] ND, not determined.

matography column (Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM sodium citrate. The antimicrobial activity was eluted by applying a 100 to 400 mM NaCl gradient in sodium citrate. Fractions of 4 ml were collected and assayed for antibacterial activity. Active fractions were subjected to peptide sequencing, using an Applied Biosystems 4774 automated sequencer. The first five amino acids (Gly-Ser-Gly-Val-Ile) were successfully identified from the amino terminus before the reactions were blocked. This total block was confirmed on at least three different sequencing runs on both variacin 8 and variacin 12 peptides. Variacin 12 was also hydrolyzed with HCl for 10 min, and three peptide fragments were isolated by high-pressure liquid chromatography (HPLC). Fragment 3 was subjected to mass spectrometry, and a mass of 540.62 was determined. The sequence of this peptide was determined to be Phe-Val-Phe-Thr, which corresponds to a calculated mass of 540.4. Variacins 8 and 12 were also subjected to mass spectrometry, which gave a molecular weight of 2,658.61 for each peptide. This indicates that the bacteriocins are most likely identical.

Computer analysis revealed a strong similarity between the amino-terminal sequence of variacin and those of the two identical bacteriocins, lacticin 481 and lactococcin DR, from *L. lactis* subsp. *lactis* strains (6, 8). Lacticin 481 starts with the amino acid sequence Lys-Gly-Gly-Ser-Gly-Val-Ile-His. The sequences are identical except for the absence of the first two residues, Lys-Gly, in variacin and the peptide sequencing block after the Ile residue. Peptide sequencing of lacticin 481, isolated from the Nestlé *L. lactis* strain SL2 (confirmed by PCR and DNA sequencing [data not shown]), was not blocked but continued after a blank residue corresponding to Abu-9, a β-methylalanyl moiety probably involved in lanthionine ring formation (6). A similarity between the peptides was also confirmed by the amino acid compositions of variacin and lacticin 481 from SL2 as determined after hydrolysis of the peptides and analysis by HPLC (Table 2). In particular, the composition indicates the presence of the same number of lanthionine and β-methyllanthionine residues for variacin and lacticin 481. Furthermore, variacin showed at least one Pro residue and differences in the number of Asx and Lys residues.

The similarity between variacin and lacticin 481 was exploited for the design of a hybridization probe for the detection of the variacin gene on genomic Southern blots of *M. varians*. Genomic DNAs of MCV8 and MCV12 were digested with several restriction enzymes (New England BioLabs, Beverly, Mass.), and the fragments were resolved on an agarose gel and transferred to a Zetaprobe nylon membrane (Bio-Rad, Richmond, Calif.). An oligonucleotide, 5′-TGGCARTTYGTNT TYACNTGYTG-3′, was designed from the internal peptide sequence Phe-Val-Phe-Thr plus the two amino acids Gln-20 and Trp-19 present in lacticin 481 and suspected to be present in variacin. The oligonucleotide was ^{32}P labelled by T4 polynucleotide kinase (Boehringer, Mannheim, Germany) and used

TABLE 2. Amino acid composition of purified variacin and lacticin 481

Amino acid	Variacin		Lacticin 481	
	% of total residues[a]	No. of residues[b]	% of total residues	No. of residues
Cys	0.0	0	0.0	0
Asp/Asn	5.4	1	10.0	2
Glu/Gln	10.8	2	9.9	2
Ser	10.2	2	9.9	2
Thr	0.9	0	0.0	0
Gly	13.2	2	15.9	3
Ala	2.7	0	1.7	0
Arg	0.8	0	0.3	0
Pro	6.0	1	0.4	0
Val	5.8[c]	2	5.7[c]	2
Met	4.5	1	4.9	1
Ile	4.2[c]	2	3.1[c]	2
Leu	1.9	0	0.9	0
Phe	12.3	3	9.7	2
Lys	0.9	0	5.0	1
His	7.7	2	9.1	2
Tyr	0.5	0	0.3	0
Lant[d]	9.8	2	11.3	2
m-Lant[e]	2.6	1	2.0	1
Total	100.2	21	100.1	23

[a] Values do not include tryptophan and were calculated from the amino acid analysis, using internal standards.
[b] Per molecule, deduced from nucleotide sequence information.
[c] Values underestimated probably due to incomplete hydrolysis of the Val-Ile bonds.
[d] Lant, lanthionine residues.
[e] m-Lant, β-methyllanthionine residues.

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101 AATCGCGGCA GCCACACCCA CTGAGAGGAC TAGAACA ATG ACG AAC GCA
                                            M   T   N   A
                                           -22     -20

150  TTT CAG GCA CTG GAC GAA GTC ACG GAC GCC GAG CTC GAC GCC
     F   Q   A   L   D   E   V   T   D   A   E   L   D   A
                -15                 -10                 -5

192  ATC CTT GGC GGG GGC AGT GGT GTT ATT CCC ACG ATC AGC CAC
     I   L   G   G   G   S   G   V   I   P   T   I   S   H
                -1  +1              5                   10

234  GAG TGC CAC ATG AAC TCC TTC CAG TTC GTG TTC ACC TGC TGC
     E   C   H   M   N   S   F   Q   F   V   F   T   C   C
                    15                  20

276  TCC TGAGAAACTC CTCCGATGCT CAGAGGGCCG
     S
     25
```

FIG. 1. DNA sequence of the region encoding the variacin gene and its deduced amino acid sequence. The numbering of the peptide sequence is adjusted to and starts with 1 for the first amino acid of the probacteriocin. The *Sac*I restriction site is underlined.

for hybridization. The autoradiogram of the hybridized blot showed the presence of specific bands: a 7-kb *Sal*I, a 1.4-kb *Sac*I, a 1.8-kb *Bam*HI, and a >15-kb *Sph*I band. To clone the variacin gene, genomic DNA of MCV8 was digested with *Bam*HI, and the 1.6- to 2.0-kb fragments were extracted from an agarose gel, ligated into pK19 (7), and transformed to *Escherichia coli* BZ234 (12). A positive clone (pK19-MCV8) was identified by PCR, using the oligonucleotide 5′-GGNTC NGGNGTNATHCAYACNATHTCNCAYGARTGYAAYA TGAA-3′, designed from the peptide sequence of the lacticin 481, as a primer and the reverse pUC19 sequencing primer (New England BioLabs). The DNA sequence of pK19-MCV8 was determined by the dideoxy sequencing method (9) and the T7 sequencing kit (Pharmacia Biotech, Dübendorf, Switzerland). The DNA sequence was compiled and analyzed with the help of the Genetics Computer Group software package (Madison, Wis.) (2). The variacin gene from MCV12 was subsequently cloned in a similar way, sequenced, and shown to be identical to that from MCV8. Comparison of the pK19-MCV8 DNA sequence with the amino-terminal peptide sequence determined for the variacin peptide identified the bacteriocin-encoding gene. The DNA sequence is shown in Fig. 1. The similarity between the variacin- and lacticin 481-encoding DNA regions is restricted to the segment encoding the bacteriocin, starting at the ATG start codon and finishing at the TAA/TGA stop codon (Fig. 2). The overall similarity is 58.7% within this 144-bp region. No DNA sequence homologies between the flanking regions of the variacin and lacticin 481 genes were detected.

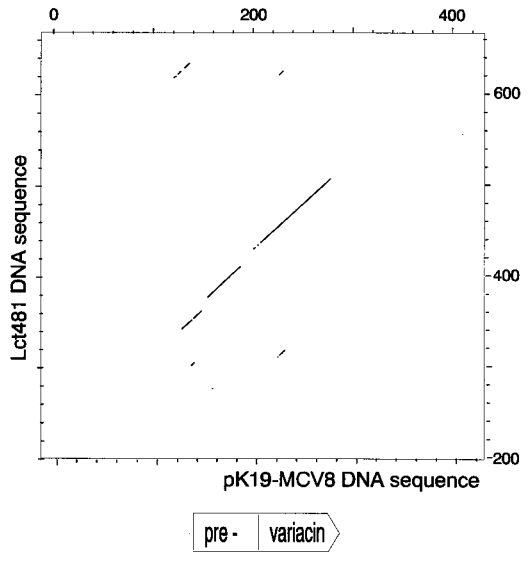

FIG. 2. Dot plot DNA sequence comparison between the *M. varians* and *L. lactis* sequence regions encoding variacin and lacticin 481, respectively. The Lct481 DNA sequence is from the EMBL data bank (accession number U04057). The comparison was done on a VAX computer with software from Genetics Computer Group Inc. (2) according to the enhanced matrix analysis of Maizel and Lenk (4). The window size was 40, and the stringency was 20 bp. The extent and orientation of the prevariacin gene are indicated below the axes of the pK19-MCV8 DNA sequence.

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                 Leader peptide sequence          Pro-peptide sequence
variacin:        MT..NAFQALDEVTDAELDAILGG ↓ ..GSGVIPTISHECHMNSFQFVFTCCS
                 |.  |.|. |:|||:.||| |||:     |||||.|||||:|||:||||||||
lacticin 481:    MKEQNSFNLLQEVTESELDLILGA   KGGSGVIHTISHECNMNSWQFVFTCCS
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FIG. 3. Alignment of the variacin and lacticin 481 prepeptide sequences. Identical residues are indicated by vertical lines; conservative changes are indicated by single or double dots according to the PAM250 scoring matrix developed by Dayhoff et al. (1). Dots within the variacin peptide sequence represent gaps. The cleavage site is indicated by a vertical arrow.

The peptide translations of the leader sequence and mature bacteriocin show different levels of homology between variacin and lacticin 481. The structural propeptide sequences share 84% identity (92% similarity), whereas the leader sequences only reveal 60% identity (75% similarity) (Fig. 3). The leader sequence of variacin is shorter by two amino acids than that of lacticin 481 and shows the same significant similarities to leader sequences of nonlantibiotic bacteriocins as lacticin 481 (3, 6). In particular, variacin revealed the presence of Gly residues in positions −2 and −1 relative to the processing site that are typically conserved for nonlantibiotic leader sequences (6). The propeptide sequences of variacin and lacticin 481 are very similar: variacin is shorter at its amino-terminal end by two amino acids and has three conservative amino acid substitutions in comparison to lacticin 481. However, the three Cys and two Ser residues and the Thr residue potentially involved in the lanthionine ring formations are conserved. The same is true for the second Thr residue, which is probably dehydrated and not detectable in the amino acid composition (Table 2).

The primary sequences and structural characteristics of the mature variacin and lacticin 481 are very similar. But the more pronounced differences of their leader sequence and their processing site and absence of homology of their flanking DNA regions may indicate that different processing and transport enzymes are used by the two bacteriocins. Hence, it may be possible that two different processing systems evolved for a similar bacteriocin in *M. varians* and *L. lactis*. However, if we assume that prevariacin and prelacticin 481 evolved from a common ancestor, we may conclude that there was clearly less selective pressure on the peptide leader to maintain its primary sequence than on the probacteriocin.

Of note is that *L. lactis* SL2, producing lacticin 481, is sensitive to variacin (Table 1), while *M. varians* MCV8 and MCV12 are both resistant to lacticin 481. The sensitivity of SL2 to variacin implies that the immunity mechanism of lacticin 481 is not (sufficiently) effective against the variacin, despite the extended primary sequence homologies between the two bacteriocins.

Nucleotide sequence accession number. The DNA sequence plus the variacin peptide translation were deposited in the EMBL data bank under accession number X93303.

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