Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A

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The strain *Enterococcus faecium* T136 produces two bacteriocins, enterocin A, a member of the pediocin family of bacteriocins, and a new bacteriocin termed enterocin B. The N-terminal amino acid sequences of enterocins A and B were determined, and the gene encoding enterocin B was sequenced. The primary translation product was a 71 aa peptide containing a leader peptide of the double-glycine type which is cleaved off to give mature enterocin B of 53 aa. Enterocin B does not belong to the pediocin family of bacteriocins and shows strong homology to carnobacteriocin A. However, sequence similarities in their leader peptides and C-termini suggest that enterocin B and carnobacteriocin A are related to bacteriocins of the pediocin family. Enterocins A and B had only slightly different inhibitory spectra, and both were active against a wide range of Gram-positive bacteria, including listeriae, staphylococci and most lactic acid bacteria tested. Both had bactericidal activities, but survival at a frequency of 10^{-4} – 10^{-2} was observed when sensitive cultures were exposed to either bacteriocin. The number of survivors was drastically reduced when a mixture of the two bacteriocins was added to the cells.

Keywords: synergistic bacteriocins, *Enterococcus faecium*

INTRODUCTION

Bacteriocins are antimicrobial proteins or peptides that are usually inhibitory only to strains closely related to the producing bacteria. Many bacteriocins from Gram-positive bacteria have fairly broad inhibitory spectra, and these bacteriocins may therefore have an applied potential as antimicrobial agents. In particular, bacteriocins from lactic acid bacteria have become attractive as natural food preservatives. Lactic acid bacteria have been used in food and feed preservation for centuries, and their preservative effects are mainly due to the formation of large amounts of lactic acid. This is not always sufficient to prevent bacterial spoilage or outgrowth of pathogenic bacteria, but bacteriocin-producing lactic acid bacteria may serve as a replacement for chemical preservatives in foods (Daeschel, 1989). The broad spectrum bacteriocins from lactic acid bacteria that have been characterized are all small (2.5–6 kDa), heat stable, hydrophobic and cationic

peptides (Klaenhammer, 1993). The best studied is nisin, which is produced by strains of *Lactococcus lactis* (Gross & Morell, 1971). This bacteriocin is approved as a food additive in many countries, and its efficiency in preventing the growth of spoilage bacteria has been proven in a number of food systems (Delves-Broughton, 1990). Another commercial bacteriocin is pediocin PA-1, produced by *Pediococcus acidilactici* PAC 1.0 (González & Kunka, 1987). This bacteriocin does not have as broad an inhibitory spectrum as nisin but it is very active against the food-borne pathogen *Listeria monocytogenes*, which is usually not very sensitive to nisin (Benkerroum & Sandine, 1988; de Vos *et al.*, 1993). Pediocin PA-1 belongs to the pediocin family of bacteriocins, identified by conserved amino acid sequences particularly in the N-terminal part of the molecule. Bacteriocins belonging to this family are produced by several genera of lactic acid bacteria, and they are all very active against listeriae (Lozano *et al.*, 1992; Aymerich *et al.*, 1996).

Application studies have, however, shown that there are limitations to the usefulness of bacteriocins as antimicrobial agents. Nisin is unstable at pH > 5, and

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although bacteriocins of the pediocin family are able to extend the shelf-life of food products, full suppression of the spoilage microflora is rarely achieved (Pucci *et al.*, 1988; Schillinger *et al.*, 1991). Most of the bacteriocins that have been studied appear to inhibit the target cells by causing permeabilization of the cell membrane. For non-lantibiotic bacteriocins such as lactococcin A and pediocin PA-1, the involvement of specific membrane-bound receptors has been inferred from the findings that liposomes are not affected (van Belkum *et al.*, 1992; Chikindas *et al.*, 1993). Complete elucidation of the primary structure of the various bacteriocins can give insight into structure-function relationships and possibly enable us to construct even more efficient bacteriocins.

In this work, we describe enterocin B. This is a new bacteriocin with a broad spectrum of inhibition that acts synergistically with enterocin A, a member of the pediocin family.

METHODS

Isolation and characterization of the bacteriocin-producing strain. Lactic acid bacteria were isolated from Spanish dry-fermented sausages, manufactured with no added starter cultures, and screened for antimicrobial activity using *Lactobacillus sake* FVM 148 and *List. monocytogenes* Scott A as indicators as described previously (Cintas *et al.*, 1995). The isolate T136 was selected for further studies because of its strong inhibitory activity against both these indicators. The strain was examined according to Schleifer & Kilpper-Bälz (1984) and further tested for arginine dihydrolase activity, Voges-Proskauer reaction (acetoin production), growth on media containing 0.04% sodium azide (Bacto E V A broth; Oxoid), urease activity and haemolysis on 5% calf-blood agar plates (Oxoid) as proposed by Devriese *et al.* (1993). Fermentation patterns were determined with API Rapid CH fermentation strips (BioMérieux). Total proteins were analysed by SDS-PAGE and the pattern obtained was compared with those of reference strains described by Kersters & de Ley (1975) and Pot *et al.* (1994) by B. Pot, University of Ghent, Belgium.

Purification of enterocins A and B. Enterocins A and B were purified from a 2 l culture of *Enterococcus faecium* T136 grown for 18 h in MRS broth (Difco) at 30 °C in a fermenter kept at pH 5.7 by the addition of 6 M sodium hydroxide. The cells were removed by centrifugation and 40 g Amberlite XAD-16 (Supelco) was added to the supernatant. The matrix was washed and the bacteriocin activity was eluted as described by Håvarstein *et al.* (1994). The eluate was subjected

to cation-exchange chromatography followed by hydrophobic interaction chromatography and reverse-phase chromatography as described by Aymerich *et al.* (1996).

Amino acid sequencing. The N-terminal amino acid sequences were determined by Edman degradation with an Applied Biosystems 477A automatic sequencer with an on-line 120A phenylthiohydantoin amino acid analyser, as described previously by Cornwell *et al.* (1988).

Mass spectrometry. Determination of the molecular mass of enterocin B was performed at the Institut für Organisch Chemie, Eberhard-Karls-Universität Tübingen, Germany, with an electrospray-ionizing mass spectrometer, API III TAGA 6000E (CIEX).

Bacteriocin assays. Bacteriocin activity was quantified using the microtitre plate assay (Holo *et al.*, 1991). Each well in the microtitre plate contained in 200 µl MRS broth (pH 6.5) bacteriocin fractions at twofold dilution and the indicator organism (10^4 times dilution of an overnight culture). The microtitre plate cultures were incubated overnight (16–20 h) at 30 °C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm by using a Dynatech Microplate Reader. One bacteriocin unit was defined as the amount of bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin) in this assay.

Minimal inhibitory concentration (MIC) values were determined using the method described by Cintas *et al.* (1995). Concentrations of purified enterocin A and enterocin B were determined spectrophotometrically at 280 nm.

The bactericidal effects of enterocin A and enterocin B were studied by the following assay. Overnight cultures of *Enterococcus faecalis* EF and *Lact. sake* NCDO 2714 were diluted 100 times in MRS. Bacteriocin was added and the cultures were incubated at 30 °C. Viable counts were determined by plating on MRS agar plates.

PCR and DNA sequencing. DNA was isolated from *E. faecium* T136 by the method of Anderson & McKay (1983). Restriction enzymes and other DNA modifying enzymes were used as recommended by the manufacturers. The sequence of the *entB* gene was determined by sequencing PCR products obtained with the primers shown in Table 1. Amplification reactions were carried out with the Gene Amp PCR kit in a DNA thermal cycler according to the supplier's (Perkin Elmer Cetus) instructions. PCR products were purified by agarose gel electrophoresis and extracted with the Qiaex II gel extraction kit (Qiagen). Primers ENTB1 and ENTB2 (see Table 1) were used in PCR to amplify a 92 bp fragment which was treated with the Klenow fragment of DNA polymerase I to generate blunt ends. Other blunt-ended DNA fragments were obtained by digesting *E. faecium* T136 DNA with *DraI*, *HincII* and *SspI*. They were all ligated to plasmid vector Bluescript SK II

Table 1. PCR primers used in this study

Primer	Sequence
ENTB1	5'-AAY GAY CAY MGN ATG CC-3'
ENTB2	5'-AAN ARN CCN CCN GCG AT-3'
ENTB3	5'-AGA CCT AAC AAC TTA TCT AAA G-3'
ENTB5	5'-GTT GCA TTT AGA GTA TAC ATT TGC-3'
ENTB6	5'-CTC CAC CGA TAA TTT GTT TCA TCT CTT TCG-3'
SK2 (biotinylated)	5'-CCG CTC TAG AAC TAG TGG ATC-3'

(Stratagene) cut with *HincII*. The ligation mixtures served as templates for the generation of biotinylated PCR products using *entB*-specific primers in combination with the biotinylated SK2 primer. Single-stranded DNA for DNA sequencing was obtained with the Dynabead M-280 Streptavidin kit and the magnetic separator Dynal-MPC-E1. The Sequenase 2.0 system (USB) was used in the sequence reactions. Analyses of DNA and protein sequences were performed using the Sequence Analysis Software Package (version 8) (Devereux *et al.*, 1984) licensed from the Genetics Computer Group, University of Wisconsin, Madison, USA.

RESULTS

Identification of *E. faecium* T136

The enterocins A and B producing isolate T136 described in this work was identified as *E. faecium* by the following criteria (Devriese *et al.*, 1993): a catalase-negative, Gram-positive, facultatively anaerobic coccus with the ability to grow at 10 and 45 °C in media containing 6.5 % NaCl, at pH 9.6, and in the presence of both 40 % (v/v) bile and 0.04 % sodium azide. It did not produce gas from glucose, gave a positive Voges-Proskauer reaction and produced ammonia from arginine. The final pH in glucose broth was 4.4, and acid was produced from ribose and L-arabinose, but not from glycogen, D-arabitol, sorbitol or gluconate. The strain did not show urease activity and it was non-haemolytic on calf-blood agar. This identification was supported by comparison of its SDS-PAGE protein pattern with a database of protein patterns from lactic acid bacteria according to Kersters & de Ley (1975) and Pot *et al.* (1994).

Characterization of enterocins A and B

The supernatant from a culture grown in a fermenter at pH 5.7 was used for the isolation of the antimicrobial compounds. The last step in the purification procedure (Table 2), reverse-phase chromatography, revealed two well-separated bacteriocin activities.

By rechromatography on the reverse-phase column, both were purified to homogeneity. Amino acid sequ-

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TGTTATTCCTCATTCAGAGTCCCAACTGTTTGCTATAGATATATGTCCTAGTATCGTA 60
ATAAAATACTCTAAAAGGAGCGAGTTTAAATATGCAAAATGTAAGAATTAAGTACGAA 120
      RBS              M Q N V K E L S T K
AGAGATGAACAAATTATCGGTGGAGAAAATGATCAGAAATGCCTAATGAGTTAAATAG 180
  E M K Q I I G G E N D H R M P N E L N R
ACCTAACAACTTATCTAAAGGTGGAGCAAAATGTGGTCTGCAATTGCTGGGGGATTATT 240
  P N N L S K G G A K C G A A I A G G L F
TGGAAATCCAAAAGGACCAGTATGATGGGCTGCTGGGTAGCAATGTATACTCTAAATG 300
  G I P K G P L A W A A G L A N V Y S K C
CAACTAAAAAGAAGAGAAAAAACTATTACGAGTTTTTTCTCTCTTTTTT 353
      N

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Fig. 1. Nucleotide sequence of the region encoding enterocin B of *E. faecium* T136 and the deduced amino acid sequence. The vertical arrow indicates the processing site of the prepeptide. A putative ribosome-binding site (RBS) is underlined. The horizontal arrows indicate a potential *rho*-independent transcription terminator sequence.

encing combined with PCR analysis showed that one of the two bacteriocins was identical to enterocin A described previously (Aymerich *et al.*, 1996), while the other was a new bacteriocin, which we have termed enterocin B. By amino acid sequencing we obtained the following sequence: ENDHRMPNELNRPNxLSKGG-AKxGAAIAGGLFGIPKGxLAW-. The molecular mass of enterocin B was determined as 5479.0 ± 1.2 Da by mass spectrometry. Enterocin B was found to be a heat-stable bacteriocin, and samples of the purified bacteriocin diluted in MRS retained 25 % of their activity after incubation at 100 °C for 20 min.

Analysis of the gene encoding enterocin B

Primers based on the partial amino acid sequence of enterocin B were constructed and PCR was used to obtain the DNA sequence of the region encoding enterocin B. Fig. 1 shows the DNA sequence of 353 contiguous nucleotides. Only one open reading frame, *entB*, was found in this sequence. The DNA sequencing

Table 2. Purification of enterocins A and B

Bacteriocin activities were determined with *P. pentosaceus* FBB 63 (enterocin A) and *Lact. sake* FVM 148 (enterocin B) as indicators.

Purification step	Enterocin A				Enterocin B			
	Vol. (ml)	A_{280}	$10^{-5} \times$ Total activity (BU)*	Yield (%)	Vol. (ml)	A_{280}	$10^{-5} \times$ Total activity (BU)*	Yield (%)
Culture supernatant	2000.0		15.0	100.0	2000.0		46	100
XAD-16	250.0		6.0	40.0	250.0		44	95
SP-Sepharose	50.0		3.0	20.0	50.0		36	79
Octyl-Sepharose	10.0		0.8	5.3	10.0		20	44
Reverse-phase	0.9	2.0	0.7	4.8	1.1	0.57	8	16

* BU, Bacteriocin unit.

Enterocin B	-	-	-	-	M	Q	N	V	K	E	L	S	T	K	E	M	K	Q	I	I	G	G	E	N	D	H	R	M	P	N	E	L	N	R	P	-	-	N	N	L	S	K	G	G	A	K	C	G	42	
Carnobacteriocin A	-	-	-	-	M	N	N	V	K	E	L	S	I	K	E	M	Q	Q	V	T	G	G	D	-	-	-	Q	M	S	D	G	V	N	Y	G	K	G	-	S	S	L	S	K	G	G	A	K	C	G	41
Acidocin A	M	I	S	M	I	S	S	H	Q	K	T	L	T	D	K	E	L	A	L	I	S	G	G	K	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	40
Bacteriocin 31	-	-	-	-	M	K	K	L	V	I	C	G	I	G	I	G	F	T	A	L	G	T	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	40
Carnobacteriocin B2	-	-	-	-	M	N	S	V	K	E	L	N	V	K	E	M	K	Q	L	H	G	G	V	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	33	
Carnobacteriocin BM1	-	-	-	-	M	K	S	V	K	E	L	N	K	K	E	M	Q	Q	I	N	G	G	A	I	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	34	
Curvacin A	-	-	-	-	M	N	N	V	K	E	L	S	M	T	E	L	Q	T	I	T	G	G	A	R	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	34	
Enterocin A	-	-	-	-	M	K	H	L	K	I	L	S	I	K	E	T	Q	L	I	Y	G	G	T	H	S	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	38	
Leucocin A	-	-	M	K	P	T	E	S	Y	E	Q	L	D	N	S	A	L	E	Q	V	V	G	G	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	36	
Pediocin PA-1	-	-	-	-	M	K	K	I	E	K	L	T	E	K	E	M	A	N	I	I	G	G	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	33	
Sakacin P	-	-	-	-	M	E	K	F	I	E	L	S	L	K	E	V	T	A	I	T	G	G	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	33	

Enterocin B	-	-	-	-	A	A	I	A	G	G	L	F	G	I	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	71
Carnobacteriocin A	-	-	-	-	L	G	I	V	G	G	L	A	T	I	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	71
Acidocin A	G	K	V	R	L	K	N	V	I	P	G	T	L	C	R	K	Q	S	L	P	I	K	Q	D	L	K	I	L	L	G	W	A	T	G	A	F	G	K	T	F	H	-	-	-	-	-	-	-	-	-	-	81	
Bacteriocin 31	-	-	-	-	V	D	W	N	K	A	S	R	E	I	G	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	67
Carnobacteriocin B2	-	-	-	-	V	N	W	G	Q	A	F	Q	E	R	Y	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	66			
Carnobacteriocin BM1	-	-	-	-	V	N	K	A	E	N	K	Q	A	I	I	T	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	61			
Curvacin A	-	-	-	-	V	N	R	G	E	A	T	Q	S	I	I	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	59			
Enterocin A	-	-	-	-	V	D	W	A	K	A	T	T	C	I	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	65			
Leucocin A	-	-	-	-	V	N	W	G	E	A	F	-	S	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	58			
Pediocin PA-1	-	-	-	-	V	D	W	G	K	A	T	T	C	I	I	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	62			
Sakacin P	-	-	-	-	V	D	W	G	T	A	I	G	N	I	G	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	61			

Fig. 2. Alignment of precursor peptides of enterocin B, carnobacteriocin A and bacteriocins of the pediocin family. The arrow indicates the processing site of the bacteriocins with double-glycine leader sequences. The possible processing site for bacteriocin 31 is indicated by ▲. Identical residues in enterocin B and carnobacteriocin A are shown in bold. Sequences were obtained from: carnobacteriocin A (Worobo *et al.*, 1994); acidocin A (Kanatani *et al.*, 1995); bacteriocin 31 (Tomita *et al.*, 1996); carnobacteriocin BM1 and B2 (Quadri *et al.*, 1994); curvacin A (Tichaczek *et al.*, 1993); enterocin A (Aymerich *et al.*, 1996), leucocin A (leucocin A-UAL 187) (Hastings *et al.*, 1991); pediocin PA-1 (Marugg *et al.*, 1992); and sakacin P (Tichaczek *et al.*, 1994).

confirmed the results of the amino acid sequencing and revealed that the bacteriocin is translated as a pre-bacteriocin of 71 aa which is then processed to give a mature enterocin B of 53 aa. The 18 aa N-terminal extension contains all the consensus elements of a leader peptide of the double-glycine type (Håvarstein *et al.*, 1994). Downstream of *entB*, two 15 bp inverted repeats separated by 5 bp can establish a stable stem-loop structure with an estimated ΔG of $-19.8 \text{ kcal mol}^{-1}$ ($-82.8 \text{ kJ mol}^{-1}$). This structure is followed by several T residues and may serve as a *rho*-independent transcription terminator (Platt, 1986).

The calculated molecular mass of the 53 aa peptide was 5465.2 Da and the pI was 9.49. The extinction coefficient at 280 nm was estimated as $7210 \text{ M}^{-1} \text{ cm}^{-1}$.

A search for similarity in the nucleotide databases revealed one bacteriocin with strong sequence similarity to enterocin B, carnobacteriocin A (Worobo *et al.*, 1994), which is identical to piscicollin 61 (Holck *et al.*, 1994). Enterocin B and carnobacteriocin A have been aligned in Fig. 2. Both bacteriocins are composed of 53 aa and are 47% identical at the amino acid level. Their leader peptides both contain 18 residues and share 72% identity.

Inhibitory spectra of enterocins A and B

The sensitivity to purified enterocin A and enterocin B of a number of organisms was investigated by a critical dilution assay. Table 3 shows the MIC values obtained

with the various test organisms used. None of the bacteriocins were inhibitory to Gram-negative bacteria in this assay (results not shown). However, both bacteriocins were inhibitory to a wide spectrum of Gram-positive bacteria, including spoilage and food-borne pathogens, such as *Clostridium sporogenes*, *Clostridium tyrobutyricum*, *Propionibacterium* spp., *List. monocytogenes* and *Staphylococcus aureus*. With only a few exceptions, their inhibitory spectra were similar, and for most of the indicators tested, lower MIC values were observed with enterocin A than with enterocin B. Some strains were only sensitive to one of the enterocins. *Pediococcus pentosaceus* FBB 63 was sensitive to enterocin A, but not to enterocin B, while *Lact. sake* FVM 148 was resistant to enterocin A but sensitive to enterocin B.

Enterocins A and B have synergistic activities

The differences seen in inhibitory spectra between enterocin A and enterocin B suggest that the two bacteriocins have different targets.

To test for synergistic activities between the two bacteriocins, mixtures of enterocin A and enterocin B were assayed in the microtitre assay. Using *Lact. sake* NCDO 2714 as indicator, we did not obtain higher titres by using mixtures than the sum of the titres of the separate bacteriocins, thus no synergism was observed in this assay (results not shown). Enterocins A and B both had bactericidal effects on cultures of sensitive

Table 3. MICs of enterocin A and enterocin B

Indicator species*	Strain	Source†	Bacteriocin concn (ng ml ⁻¹)	
			Enterocin A	Enterocin B
<i>Lactobacillus sake</i>	2714	NCDO	0.1	0.8
<i>Lactobacillus sake</i>	148	FVM	NID	0.4
<i>Lactobacillus curvatus</i>	2739	NCFB	480	NID
<i>Lactobacillus fermentum</i>	9338	ATCC	810	34
<i>Pediococcus pentosaceus</i>	FBB63	TNO	610	NID
<i>Enterococcus faecalis</i>	EF	TNO	11	8
<i>Clostridium sporogenes</i>	C22/10	TNO	1000	460
<i>Clostridium tyrobutyricum</i>	3,5CT	TNO	1400	550
<i>Clostridium tyrobutyricum</i>	1754	NCDO	380	140
<i>Clostridium perfringens</i>	376	CECT	NID	NID
<i>Clostridium botulinum</i>	551	CECT	NID	NID
<i>Propionibacterium</i> sp.	P4	TNO	50	90
<i>Propionibacterium</i> sp.	P6	TNO	40	50
<i>Propionibacterium acidipropionici</i>	573	NCDO	40	90
<i>Listeria monocytogenes</i>	7973	NCTC	40	150
<i>Listeria monocytogenes</i>	LI5sv1/2	FVM	100	440
<i>Listeria monocytogenes</i>	5105	NCTC	50	200
<i>Listeria monocytogenes</i>	LI1sv4	FVM	40	160
<i>Listeria monocytogenes</i>	Scott A	FVM	40	140
<i>Listeria innocua</i>	BL86/26	TNO	120	430
<i>Staphylococcus aureus</i>	137	FRI	530	460
<i>Staphylococcus aureus</i>	196E	FRI	580	1000
<i>Staphylococcus aureus</i>	472	FRI	1300	770
<i>Staphylococcus carnosus</i>	MC1	TNO	180	410

NID, No inhibition detected at 3.75 µg enterocin A ml⁻¹ or 5.0 µg enterocin B ml⁻¹.

* Growth media for indicator species: lactic acid bacteria strains, MRS medium (Difco); *Clostridium* strains, RCM (Oxoid); *Propionibacterium* spp., GYE medium (Cintas *et al.*, 1995); other strains, BHI medium (Oxoid).

† Source abbreviations: ATTC, American Type Culture Collection (Rockville, MD, USA); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); NCDO and NCFB, National Collection of Food Bacteria (Reading, UK); NCTC, National Collection of Type Cultures (London, UK); TNO, Nutrition and Food Research (Zeist, The Netherlands); FVM, Facultad de Veterinaria (Madrid, Spain); FRI, Food Research Institute (Madison, WI, USA).

strains (Fig. 3). Despite this, a low number of cells survived and were able to grow in the presence of either bacteriocin. However, mutants resistant to one of the two bacteriocins were still sensitive to the other (results not shown). We compared the bactericidal effect of enterocins A and B and mixtures of them. The number of survivors was drastically reduced in cultures treated with both bacteriocins compared to cultures exposed to only enterocin A or B, demonstrating a synergistic effect between the two bacteriocins. In the experiments described in Fig. 3, no survival was observed when a mixture of enterocins A and B was used.

DISCUSSION

During the last few years, a large number of bacteriocins from lactic acid bacteria have been described at the molecular level (Klaenhammer, 1993). Most are small

(2.5–6 kDa), thermostable, hydrophobic and cationic peptides, and enterocin B falls into this category. Our results indicate that enterocin B does not contain modified amino acids. The molecular mass found by mass spectrometry was 14 Da higher than that calculated from the amino acid sequence. This difference can be ascribed to oxidation of the only methionine residue in the bacteriocin and the formation of a disulfide bridge between its two cysteine residues. These modifications are formed spontaneously and have also been observed with synthetic peptide bacteriocins (Fimland *et al.*, 1996). When assayed in the presence of 5 mM dithiothreitol to reduce disulfide bridges, the titre of enterocin B was half of that found in the absence of the thiol compound (results not shown). This finding supports the idea of a disulfide bridge between Cys-23 and Cys-52 in enterocin B. Similar experiments have been done with pediocin PA-1 (Chikindas *et al.*, 1993) and colicin V

Fig. 3. Killing of *E. faecalis* EF (a) and *Lact. sake* NCDO 2714 (b) with purified enterocin A and enterocin B. (a) □, Enterocin A (41 ng ml⁻¹); ◇, enterocin B (190 ng ml⁻¹); ■, enterocin A (21 ng ml⁻¹) + enterocin B (95 ng ml⁻¹); ●, no bacteriocin. (b) □, Enterocin A (16 ng ml⁻¹); ◇, enterocin B (173 ng ml⁻¹); ■, enterocin A (8 ng ml⁻¹) + enterocin B (87 ng ml⁻¹); ●, no bacteriocin.

(Håvarstein *et al.*, 1994). In both these cases, the activity was decreased when the Cys residues were in the reduced state.

Despite their physico-chemical similarities, there is a remarkable lack of sequence similarity among most of the bacteriocins from lactic acid bacteria (Klaenhammer, 1993). The pediocin family is the only grouping that has been identified by sequence similarities, and bacteriocins of this family all contain the motif YGNGVxC in the N-terminal part (Lozano *et al.*, 1992; Aymerich *et al.*, 1996). Enterocin B does not contain this motif. However, homology was found to carnobacteriocin A (Worobo *et al.*, 1994), also described as piscicolin 61 (Holck *et al.*, 1994). Enterocin B and carnobacteriocin A are 47% identical. To our knowledge, this is the first report on two non-lantibiotic bacteriocins from lactic acid bac-

teria with strong sequence similarity outside the pediocin family of bacteriocins.

The gene encoding bacteriocin immunity is usually located immediately downstream of, and in the same operon as, the structural gene for non-lantibiotics of lactic acid bacteria. Enterocin B and carnobacteriocin A are exceptions to this rule. In both cases, the bacteriocin gene is followed by a putative *rho*-independent terminator. Genes encoding immunity to enterocin B or carnobacteriocin A have not been identified, but according to Worobo *et al.* (1994) the genes for carnobacteriocin A production and immunity are located on the same 5.4 kb restriction fragment of the bacteriocin plasmid.

Despite the absence of the YGNGVxC motif, enterocin B shares sequence similarities with members of the pediocin family. Its 18 aa leader peptide has the same size as, and 50% or more sequence identity to, the leader peptide of carnobacteriocin A and most members of the pediocin family (Fig. 2). Furthermore, the 16 C-terminal amino acids of pediocin PA-1 are 42% identical (54% similar) to the corresponding residues in enterocin B. The sequence alignment of the prepeptides of enterocin B, carnobacteriocin A and pediocin-like bacteriocins revealed that consensus residues, including a GWAxG motif, are shared between the two groups of bacteriocins in their C-terminal parts.

It is tempting to speculate that the conserved residues in the C-terminal region reflect structure-function relationships. Fimland *et al.* (1996) showed that the C-terminal part of the pediocin-like bacteriocins is of importance for their activity. It is interesting to note that leucocin A-UAL 87, which lacks some of the consensus residues in the C-terminal part of the molecule, has bacteriostatic but no bactericidal activity (Stiles, 1994).

Enterocins A and B both have broad and very similar inhibitory spectra. From an applied point of view, it is interesting that both are able to inhibit food-borne and pathogenic species like *List. monocytogenes*, *C. tyrobutyricum* and *S. aureus*. Since enterocin B was inhibitory to a few strains that were insensitive to enterocin A, we hypothesized that the two bacteriocins could have differences in their mode of action and therefore may act synergistically. Two assays were used to test for synergism. In the critical dilution assay, the bacteriocin concentration causing 50% growth inhibition can be determined, but the level of inhibition at other bacteriocin concentrations is not determined. No synergism between enterocins A and B was observed in this assay. Because the bactericidal effect of neither bacteriocin was complete, we could investigate synergism at higher bacteriocin concentrations by measuring the survival of cells exposed to the bacteriocins. At concentrations at which most of the cells were killed, the synergistic bactericidal effects of enterocins A and B were clearly demonstrated, and the number of survivors was reduced by several orders of magnitude when a mixture of the two bacteriocins was used.

Typically, a 3–4 log reduction in c.f.u. is obtained by exposing a sensitive culture to pediocin-like bacteriocins (Chikindas *et al.*, 1993; Rekhif *et al.*, 1994; Schillinger *et al.*, 1991). The survivors from exposure to high concentrations of bacteriocins belonging to the pediocin family remain resistant to the bacteriocin (Rekhif *et al.*, 1994), but the survivors of exposure to enterocin A had not acquired resistance to enterocin B (results not shown). Thus a likely explanation of the reduced survival obtained by exposing a culture to both bacteriocins is that enterocin B is able to kill the bacteria that survive enterocin A treatment.

Our findings may be important from an applied point of view. In food preservation, the combined use of enterocin A and enterocin B is likely to be better than using a pediocin-like bacteriocin alone to prevent growth of spoilage bacteria. As a starter culture for food fermentation, *E. faecium* T136 has the advantage that it produces both these bacteriocins.

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REFERENCES

- Anderson, D. G. & McKay, L. L. (1983). Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl Environ Microbiol* **46**, 549–552.
- Aymerich, T., Holo, H., Håvarstein, L. S., Hugas, M., Garriga, M. & Nes, I. F. (1996). Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Appl Environ Microbiol* **62**, 1676–1682.
- van Belkum, M. J., Kok, J., Venema, G., Holo, H., Nes, I. F., Konings, W. N. & Abee, T. (1992). The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. *J Bacteriol* **173**, 7934–7941.
- Benkerroum, N. & Sandine, W. E. (1988). Inhibitory action of nisin against *Listeria monocytogenes*. *J Dairy Sci* **71**, 3237–3245.
- Chikindas, M. L., García-Garcera, M. J., Driessen, A. J. M., Ledebøer, A. M., Nissen-Meyer, J., Nes, I. F., Abee, T., Konings, W. N. & Venema, G. (1993). Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Appl Environ Microbiol* **59**, 3577–3584.
- Cintas, L. M., Rodríguez, J. M., Fernández, M. F., Sletten, K., Nes, I. F., Hernández, P. E. & Holo, H. (1995). Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Appl Environ Microbiol* **61**, 2643–2648.
- Cornwell, G. G., Sletten, K., Johansson, B. & Westermarck, P. (1988). Evidence that the amyloid fibril protein in senile systemic amyloidosis is derived from normal prealbumin. *Biochem Biophys Res Commun* **154**, 648–653.
- Daeschel, M. A. (1989). Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol* **43**, 164–167.
- Delves-Broughton, J. (1990). Nisin and its use as a food preservative. *Food Technol* **44**, 100–117.
- Devereux, J. P., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Devriese, L. A., Pot, B. & Collins, M. D. (1993). Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* **75**, 399–408.
- Fimland, G., Blingsmo, O. R., Sletten, K., Jung, G., Nes, I. F. & Nissen-Meyer, J. (1996). New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region is important for determining specificity. *Appl Environ Microbiol* **62**, 3313–3318.
- González, C. F. & Kunka, B. S. (1987). Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. *Appl Environ Microbiol* **53**, 2534–2538.
- Gross, E. & Morell, J. L. (1971). The structure of nisin. *J Am Chem Soc* **93**, 4634–4635.
- Hastings, J. W., Sailer, M., Johnson, K., Roy, K. L., Vederas, J. C. & Stiles, M. E. (1991). Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. *J Bacteriol* **173**, 7491–7500.
- Håvarstein, L. S., Holo, H. & Nes, I. F. (1994). The leader peptide of colicin V shares consensus sequences with leader peptides that are common amongst peptide bacteriocins produced by Gram-positive bacteria. *Microbiology* **140**, 2383–2389.
- Holck, A., Axelsson, L. & Schillinger, U. (1994). Purification and cloning of piscicolin 61, a bacteriocin from *Carnobacterium piscicola* LV61. *Curr Microbiol* **29**, 63–68.
- Holo, H., Nilssen, Ø. & Nes, I. F. (1991). Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. *J Bacteriol* **173**, 3879–3887.
- Kanatani, K., Oshimura, M. & Sano, K. (1995). Isolation and characterization of acidocin A and cloning of the bacteriocin gene from *Lactobacillus acidophilus*. *Appl Environ Microbiol* **61**, 1061–1067.
- Kerstens, K. & de Ley, J. (1975). Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. *J Gen Microbiol* **87**, 333–342.
- Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**, 224–227.
- Lozano, J. C. N., Nissen-Meyer, J., Sletten, K., Peláez, C. & Nes, I. F. (1992). Purification and amino acid sequence of a bacteriocin produced by *Pediococcus acidilactici*. *J Gen Microbiol* **138**, 1985–1990.
- Marugg, J. D., González, C. F., Kunka, B. S., Ledebøer, A. M., Pucci, M. J., Toonen, M. Y., Walker, S. A., Zoetmulder, L. C. M. & Vandenberg, P. A. (1992). Cloning, expression and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC 1.0. *Appl Environ Microbiol* **58**, 2360–2367.

- Platt, T. (1986).** Transcription termination and regulation of gene expression. *Annu Rev Biochem* **55**, 1822–1828.
- Pot, B., Vandamme, P. & Kersters, K. (1994).** Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: J. Wiley.
- Pucci, M. J., Vedamuthu, E. R., Kunka, B. S. & Vandenberg, P. A. (1988).** Inhibition of *Listeria monocytogenes* by using bacteriocin pediocin PA-1 produced by *Pediococcus acidilactici* PAC1.0. *Appl Environ Microbiol* **54**, 2349–2353.
- Quadri, L. E. N., Sailors, M., Roy, K. L., Vederas, J. C. & Stiles, M. E. (1994).** Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. *J Biol Chem* **269**, 12204–12211.
- Rekhif, N., Atrih, A. & Lefebvre, G. (1994).** Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. *Curr Microbiol* **28**, 237–241.
- Schillinger, U., Kaya, M. & Lücke, F. K. (1991).** Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin producing strain of *Lactobacillus sake*. *J Appl Bacteriol* **70**, 473–478.
- Schleifer, K. H. & Kilpper-Bälz, R. (1984).** Transfer of *Streptococcus faecalis* and *S. faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J Syst Bacteriol* **34**, 31–34.
- Stiles, M. E. (1994).** Bacteriocins produced by *Leuconostoc* species. In *Bacteriocins of Lactic Acid Bacteria. Microbiology, Genetics and Applications*, pp. 497–506. Edited by L. DeVuyst & E. C. Vandamme. London: Blackie Academic and Professional.
- Tichaczek, P. S., Vogel, R. F. & Hammes, W. P. (1993).** Cloning and sequencing of *curA* encoding curvacin A, the bacteriocin produced by *Lactobacillus curvatus* LTH1174. *Arch Microbiol* **160**, 279–283.
- Tichaczek, P. S., Vogel, R. F. & Hammes, W. P. (1994).** Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. *Microbiology* **140**, 361–367.
- Tomita, H., Fujimoto, S., Tanimoto, K. & Ike, Y. (1996).** Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. *J Bacteriol* **78**, 3585–3593.
- de Vos, W. M., Mulders, J. W. M., Siezen, R. J., Hugenholtz, J. & Kuipers, O. P. (1993).** Properties of nisin Z and distribution of its gene, *nisZ*, in *Lactococcus lactis*. *Appl Environ Microbiol* **59**, 213–218.
- Worobo, R. W., Henkel, T., Sailer, M., Roy, K. L., Vederas, J. C. & Stiles, M. E. (1994).** Characteristics and genetic determinant of a hydrophobic peptide bacteriocin, carnobacteriocin A, produced by *Carnobacterium piscicola* LV17A. *Microbiology* **140**, 517–526.

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