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Isolation and characterization of two bacteriocins of *Lactobacillus acidophilus* LF221

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Abstract *Lactobacillus acidophilus* LF221 produced bacteriocin-like activity against different bacteria including some pathogenic and food-spoilage species. Besides some lactic acid bacteria, the following species were inhibited: *Bacillus cereus*, *Clostridium* sp., *Listeria innocua*, *Staphylococcus aureus*, *Streptococcus* D. *L. acidophilus* LF221 produced at least two bacteriocins, acidocin LF221 A and acidocin LF221 B, which were purified by ammonium sulphate precipitation, ion-exchange chromatography, hydrophobic interaction and reverse-phase FPLC. The antibacterial substances were heat-stable, sensitive to proteolytic enzymes (trypsin, pepsin, pronase, proteinase K) and migrated as 3500- to 5000-Da proteins on sodium dodecyl sulphate/polyacrylamide gel electrophoresis. The sequences of 46 amino-terminal amino acid residues of peptide A and 35 of peptide B were determined. Among the residues identified, no modified amino acids were found. No significant homology was found between the amino acid sequences of acidocin LF221 A and other bacteriocins of lactic acid bacteria and 26% homology was found between acidocin LF221 B and brevicin 27. *L. acidophilus* LF221 may be of interest as a probiotic strain because of its human origin and inhibition of pathogenic bacteria, especially *Clostridium difficile*.

Introduction

Lactic acid bacteria produce a variety of antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins. Bacteriocins are proteinaceous compounds with a bactericidal mode of action, produced by a variety of micro-organisms (Tagg et al. 1976). Beside typical bacteriocins with a narrow antibacterial spectrum, a few bacteriocins of lactic acid bacteria with a wider spectrum have also been described (Klaenhammer 1993; Jack et al. 1995). On the basis of their biochemical characteristics, bacteriocins of lactic acid bacteria have been classified into four main groups (Klaenhammer 1993). The majority of bacteriocins of *Lactobacillus* species belong to the class II bacteriocins, which are small, heat-stable and hydrophobic and are synthesised as precursors. Bacteriocin production is quite widespread among *Lactobacillus acidophilus* and related species of lactobacilli, such as *L. gasseri* and *L. johnsonii*, and detailed information about their genetic material and proteins has been reported for some bacteriocins. Lactacin F was the first biochemically and genetically characterized bacteriocin of a *L. acidophilus* strain, which was later reclassified to *L. johnsonii* (Muriana and Klaenhammer 1987, 1991a, 1991b; Fujisawa et al. 1992). Lactacin F is actually a two-component bacteriocin, active against related lactobacilli and *Enterococcus faecalis* (Fremaux et al. 1993; Allison et al. 1994). The acidocin B inhibitory spectrum also includes some non-lactic-acid bacteria, such as *Clostridium sporogenes*, *Listeria monocytogenes* and *Brochothrix thermosphacta* (ten Brink et al. 1994; van der Vossen et al. 1994). Acidocin A is another *L. acidophilus* bacteriocin with an interesting spectrum of activity, including the inhibition of some food-borne pathogens, among them *Listeria monocytogenes* (Kanatani et al. 1995). Recently, acidocin J1132, a two-component bacteriocin with almost identical components and with a narrow spectrum of activity, has been described (Tahara et al. 1996).

The possible use of the broad-spectrum bacteriocins of lactic acid bacteria as food preservatives is becoming increasingly interesting, as many of them are able to inhibit the growth of food-contaminating bacteria (Nettles and Barefoot 1993; De Vuyst and Vandamme

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1994). In addition, the probiotic characteristics of lactobacilli from the intestinal tract have often been cited. *L. acidophilus* strains especially are believed to have colonization and competitive abilities in the intestinal tract. Many of them also produce bacteriocins, but the role of bacteriocins in the interactions in the intestinal tract of humans and animals has been little investigated so far (Klaenhammer 1988; Tannock 1990; Alm 1991; Toba et al. 1991; De Vuyst and Vandamme 1994).

In the present study, two new bacteriocins produced by *L. acidophilus* isolated from child's faeces were characterized and the N-terminal amino acid sequences of both bacteriocins determined. *L. acidophilus* LF221 has been previously found to exhibit bacteriocinogenic activity against certain lactic acid bacteria and also against the members of some other genera (Bogovič-Matijašić and Rogelj 1995). The strain could be interesting as a potential probiotic strain.

Materials and methods

Bacterial strains and media

The strain LF221 (ZIM BI50, Collection of Industrial Microorganisms, Ljubljana, Slovenia) was isolated from infant faeces at Istituto di Microbiologia, Facoltà di Agraria, Università Cattolica dal Sacro Cuore, Piacenza, Italia. It was propagated in MRS broth (De Man et al. 1960) at 37 °C. Stock cultures were stored in MRS broth with 20% glycerol at −20 °C and in liquid nitrogen. The strain LF221 was identified on the basis of Gram staining, the catalase test, its carbohydrate fermentation pattern, production of L- and D-lactic acid, G+C content (mol%) and DNA-homology studies. The API 50CHL system was used for biochemical tests according to the manufacturer's instructions (API System, La Balme Les Grottes, France). The amount of LD-lactic acid, produced by strain LF221 in the MRS broth was determined by a kit for enzymatic determination (Boehringer-Mannheim). The G+C content was calculated from the thermal denaturation analysis of DNA by the spectrophotometer Response II Gilford Tm Programmer (De Ley 1970). The methods described by Dellaglio et al. (1973) were used to determine the DNA homology.

Bacterial strains used as target strains are listed in Table 1.

Detection of antibacterial activity

The strain LF221 was screened for a bacteriocin-like inhibition of different bacteria by a deferred agar-spot assay (Tagg et al. 1976) and an agar-well diffusion method (Klaenhammer 1988). A 5-µl sample of an 18-h MRS culture of the strain LF221 was spot-inoculated on the surface of dried M17 agar and grown for 24 h at 37 °C. The plates were overlaid with 3 ml MRS, M17, BHI or RCM soft agar (0.75% agar), inoculated with 10^7 cells of the target strain in stationary phase. Inhibition zones were observed after 24–48 h of incubation.

For the agar-well assay, the overlay agar was prepared as described above and poured onto prepoured MRS and M17 agar plates. Wells of 1.5 mm diameter were cut into the agar and filled with 20 µl culture extract. The plates were left for 4 h at room temperature and then incubated at a temperature suitable for a particular indicator strain. After 24–48 h the plates were examined for zones of inhibition. A bacteriocin preparation was made from the cell-free supernatant of MRS cultures. After the cells of *L. acidophilus* LF221 had been removed from a 24-h culture by centrifugation at 3500 *g* for 10 min, the supernatant was concentrated 20-fold by ultrafiltration with a Minitan S unit (Millipore) with 10 K polysulphone ultrafiltration sheet. The concentrated supernatant was adjusted to pH 6.8 by 5 mol l^{-1} NaOH and sterilized by passage through a 0.22-µm filter (Sigma). Filter-sterilized catalase from bovine liver (Sigma), dissolved in 50 mmol l^{-1} sodium-phosphate buffer, was added at a final concentration of 100 U ml^{-1}. As a control, MRS broth was concentrated 20-fold by ultrafiltration.

Bacteriocin activity assay

Lactobacillus sake NCDO 2714 (National Collection of Dairy Organisms, Reading, England) was used as an indicator strain. A critical-dilution method was used as described by Mortvedt and Nes (1990) and Holo et al. (1991) and the following adaptation was used for our strain. Each cell on the microtitre plate contained 50 µl MRS broth, 1 µl 2-fold serial dilutions of the sample with bacteriocin and 150 µl overnight *L. sake* NCDO 2714 culture in MRS broth, diluted 10 000-fold. Bacteriocin dilutions were made directly into MRS broth on microtitre plates. The microtitre plates were incubated for 18 h at 30 °C and absorbance was measured at 630 nm. One bacteriocin unit was defined as the amount of bacteriocins causing 50% growth inhibition, compared with a control without bacteriocins.

Sensitivity of bacteriocins to trypsin, heat and pH value

A neutralized and 10-fold concentrated cell-free supernatant of *L. acidophilus* LF221 of pH 6.8 (bacteriocin preparation) was treated with trypsin or heat, and the pH was adjusted to different values. The residual bacteriocin activity was determined by the microtitre plate assay.

Enzymes

Aliquots of 500 ml bacteriocin preparation were treated with trypsin at concentrations of 0.1 mg ml^{-1}, 1 mg ml^{-1}, 5 mg ml^{-1} and 10 mg ml^{-1} at 37 °C for 1 h. An untreated culture extract and trypsin in the buffer alone served as controls.

Heat

Aliquots of 500 ml bacteriocin preparation were heat-treated at 115 °C for 20 min, 70 °C for 30 min, 70 °C for 60 min, 100 °C for 5 min, 100 °C for 15 min and 100 °C for 30 min and then quickly chilled in ice water.

pH

Aliquots of 10 ml bacteriocin preparation were adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9 and 10 with 5 mol l^{-1} NaOH and with concentrated HCl. Tenfold-concentrated MRS broth, pH 2–10, served as a control.

SDS-PAGE electrophoresis

The method of Schägger and von Jagow (1987) was used for sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE). The gel was composed of 4% acrylamide and 0.5% bisacrylamide in the "stacking" gel and 16.5% acrylamide and 0.5% bisacrylamide in the "separating" gel. Electrophoresis was at a constant current of 35 mA in the "stacking" gel and 50 mA till the end. A 15-µl aliquot of the 20-fold concentrated bacteriocin sample was mixed with 15 µl 2-fold-concentrated sample buffer and boiled for 5 min. Two molecular-mass standards were applied: MW-17 (Sigma), 2510–16 950 Da. After electrophoresis, the gel was divided into two parts. The first half, with standards and bacteriocin sample, was stained with Coomassie R250 stain. The other part contained only bacteriocin sample and was used for

activity detection. It was placed in sterile mQ water for 24 h, with frequent changing of water, and then overlaid with the indicator strain *Lactobacillus helveticus* ATCC 15009 (American Type Culture Collection, Rockville, USA). After overnight incubation, the position of the inhibition zone was detected and compared with the stained part.

Purification of bacteriocins

The strain LF221 was grown in 2100 ml MRS broth in a fermentor with a pH controller, under the optimal conditions for bacteriocin production determined previously (Bogovič-Matijašić and Rogelj 1998): 18 h at 37 °C, constant pH 6.5 and under N_2 atmosphere. During fermentation, the pH was controlled by the addition of 5 mol l^{-1} NaOH solution. The cells were removed by centrifugation at 4000 *g* for 15 min at 4 °C. Proteins were precipitated by addition of 300 *g* ammonium sulphate l culture supernatant^{-1}, followed by centrifugation at 7000 *g* for 20 min. The pellet was resuspended in 400 ml 5 mmol l^{-1} sodium phosphate buffer pH 5 (fraction I), and applied to a 10-ml S-Sepharose Fast-Flow cation-exchange column, equilibrated with 5 mmol l^{-1} sodium phosphate buffer, pH 5. The activity was eluted with 50 ml 1 mol l^{-1} NaCl in 5 mmol l^{-1} sodium phosphate buffer pH 5 (fraction II). Fraction II was applied to an octyl-Sepharose column, equilibrated with 1 mol l^{-1} NaCl in 5 mmol l^{-1} sodium phosphate buffer pH 5 and bacteriocin activity was eluted with 10 ml 70% (v/v) ethanol (fraction III). Fraction III was further purified by reverse-phase chromatography on a C_2/C_{18} column, PepRPC HR 5/5, equilibrated with 0.1% (v/v) trifluoroacetic acid (CF_3COOH) in distilled water. Bacteriocins were eluted with a linear gradient ranging from 20% to 40% 2-propanol containing 0.1% CF_3COOH. Active fractions were diluted 4- to 5-fold in 0.1% (v/v) CF_3COOH and rechromatographed (a linear gradient was 30%–50% v/v 2-propanol and 0.1% v/v CF_3COOH). Two active fractions were rechromatographed separately in a narrower linear gradient (30%–40% v/v 2-propanol and 0.1% v/v CF_3COOH. The FPLC system (Pharmacia-LKB Biotechnology, Uppsala, Sweden) was used for the reverse-phase chromatography.

Amino acid sequencing

The NH_2-terminal amino acid sequences were determined by Edman degradation using an Applied Biosystems (Foster City, Calif.) 477A automatic sequence analyzer with an on-line 120A amino acid phenylthiohydantoin analyzer as described previously (Cornwell et al. 1988; Kok et al. 1993). The amino acid sequences were compared with the others of the NCBI database using the Blast program (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast).

Results

Identification of strain LF221

The cells of strain LF221 are gram-positive and catalase-negative rods. In MRS broth at 37 °C the strain produced 36% L(+)- and 64% D(−)-lactic acid. The carbohydrate fermentation pattern determined with the API 50 CHL system (Bio-Merieux, France) most closely resembled that of *L. acidophilus*. As determined by DNA melting analysis, it contained 37 mol% G+C. The percentage of DNA homology with DNA of the type strain of *L. acidophilus* species was 86%. On the basis of these results the strain LF221 was identified as *L. acidophilus*.

Inhibitory spectrum

The first screening by the deferred agar spot method was performed on MRS agar plates (results not shown), but MRS agar was found not to be a suitable medium, because almost all the tested strains were inhibited to some extent, probably because of the lactic acid, and no inhibition zones with clear edges were observed. The results in Table 1 were obtained with the deferred agar-spot assay (DAS) on M17 agar and with the agar-well diffusion assay (AWD). Only the sensitive strains are shown in Table 1.

Some differences between the results obtained by the two methods were observed: 9 strains from different species were found to be sensitive to concentrated MRS supernatant of *L. acidophilus* LF221, as shown by the AWD test, although the DAS test on M17 agar or application of non-concentrated MRS supernatant did not result in an inhibition zone. The inhibition zones around the spots on M17 agar were not greater than 2 mm in most cases, except in the case of the clostridial strains with zones of 5–8 mm. The inhibition zones on MRS agar were greater, from 3 mm to 15 mm (results not shown). The least sensitive strains only showed inhibition zones in the AWD assay, possibly because more bacteriocin was present in this assay.

The concentrated supernatant of the M17 culture of strain LF221 was also tested by the AWD assay, but it had to be approximately five-times as concentrated as the MRS culture supernatant to obtain the comparable inhibition zones. Less bacteriocin were produced in M17 broth (1.170 *A* units/ml) than in MRS (6.240 *A* units/ml), although the cell masses in MRS and in M17 broth were comparable (results not shown).

Of the gram-negative strains tested, 39 were not inhibited (results not shown). From 10 species of lactobacilli tested, only *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus* and *L. sake* were inhibited. It is interesting that all tested strains of the genus *Clostridium*, except for five *Clostridium perfringens* strains, were inhibited with the concentrated supernatant. *Clostridium tyrobutyricum* and *Clostridium sporogenes* were the most sensitive strains and were inhibited also by the DAS test. The strains *Clostridium feseri* and *Clostridium septicum* were isolated from cattle, *Clostridium difficile* strains were human isolates. Inhibition of *Cl. difficile* strains is of particular interest because they are pathogenic. Although *Bacillus cereus* ATCC 9139 was inhibited, the other 11 strains of the same species, isolated from food and water, were not.

Sensitivity of bacteriocins to proteases, heat and pH value

The sensitivity of bacteriocins to trypsin is shown in Table 2. Pepsin, proteinase K and pronase also inactivated bacteriocins in the liquid and in the solid media (results not shown). Catalase did not affect the inhibi-

Table 1 Inhibitory spectrum of the strain *Lactobacillus acidophilus* LF221, determined on M17 agar plates by deferred agar-spot test and by agar-well diffusion assay. Media are those used for cultivation of indicator strains and for soft agar used in the agar-spot test and in the agar-well assay. *DAS* inhibition of indicator strains by deferred agar-spot test; the spots were made from 5- or 18-h MRS culture of the strain LF221; data represent the distance between the edge of the spot (strain LF221) and the outer edge of the inhibition zone; – no inhibition: 1 mm or less. *AWD* inhibition of indicator strains by agar-well diffusion assay; 20 µl 20-fold concentrated and neutralized supernatant from the strain LF221 culture was added to the wells; data represent the radius of the inhibition zone; the radius of the well (0.75 mm) is subtracted. *ATCC* American Type Culture Collection, Rockville, USA; *NCDO* National Collection of Dairy Organisms, National Institute for Dairying, Reading, England; *TNO* Nutrition and Food Research, Zeist, the Netherlands; *ZIM* Collection of Industrial Microorganisms, Ljubljana, Slovenia

Indicator strain	Source	Medium	Temperature (°C)	Inhibition	
				DAS (mm)	AWD (mm)
Bacillus cereus ATCC 9139	ATCC	BHI	37	–	3
Clostridium sporogenes CS 22/10[b]	TNO	RCM	37	8	5
Cl. tyrobutyricum ZIM BI03	TNO	RCM	37	5	4
Cl. tyrobutyricum CT[b] NCDO 1754	NCDO	RCM	30	5	2.5
Cl. difficile[b] (7 strains: ZIM BI53, 54, 55, 56, 57, 58, 59)	ZIM	RCM	30	–	3
Cl. septicum[b] ZIM BI61	ZIM	RCM	37	–	3
Cl. feseri[b] ZIM BI62	ZIM	RCM	37	–	3
Enterococcus faecalis EF	TNO	BHI	37	1.5	4
Lactobacillus acidophilus[a] ATCC 4356	ATCC	MRS	37	1.5	3
Lact. delbrueckii subsp. *bulgaricus*[a] ATCC 11842	ATCC	MRS	42	1.5	4
L. helveticus[a] ATCC 15009	ATCC	MRS	42	2.0	4
L. sake[a] NCDO 2714	NCDO	MRS	30	1.5	4
L. sake L45[a]	Mortvedt and Nes (1990)	MRS	30	1.5	3
Lactococcus lactis subsp. *lactis*[a] ZIM BI51	ZIM	M17	30	–	2.5
L. lactis subsp. *cremoris* CNRZ 117	TNO	M17	30	–	2.5
Listeria innocua BL 86/26	TNO	BHI	37	2	3
Pediococcus pentosaceus FBB 63[a]	TNO	M17	30	–	2
Ped. pentosaceus PC 1[a]	TNO	M17	30	–	2
Staphylococcus aureus SA 113	TNO	BHI	37	–	2
Streptococcus sp. ZIM BI60	ZIM	BHI	37	2	3
Strep. thermophilus ST 112[a]	TNO	M17	42	2	3.5
Strep. thermophilus ST 20[a]	TNO	M17	42	2	3.5
Strep. thermophilus[a] (4 strains: ZIM BI09, 10, 11, 12)	ZIM	M17	42	2	3–4

[a] Incubated in microaerophilic atmosphere (Biomerieux, Generbag, Generbox)
[b] Incubated in anaerobic atmosphere (Biomerieux, Generbag, Generbox)

tion. The activity of bacteriocins was not affected by heating at 100 °C for 5 min and remained unchanged at a wide range of pH values, from 2 to 9. It was reduced at pH 10, but still present (results not shown).

Determination of molecular mass by gel filtration and SDS-PAGE

During the gel-filtration chromatography performed on the Sephadex G100 column, almost all the activity was eluted at a void volume (blue dextran elution volume), indicating that most of bacteriocins were in the form of aggregates with a molecular mass of 150 kDa or more. All the activity was retained by the membranes with molecular mass exclusion limits 10 kDa and 100 kDa during ultrafiltration.

After SDS-PAGE electrophoresis, the bacteriocin activity was detected as an inhibition zone, spread at the position of 3.5–5 kDa, as determined from the comparison with the molecular mass marker MW-17 (Sigma). When the same amount of partially purified bacteriocin preparation that was sufficient for detection of activity (30 µl) was applied on the part of the gel that was stained after electrophoresis, no visible bands were observed at the position of the inhibition zone. Higher concentrations of the sample (150 µl) had to be applied to produce a diffused band where expected on the basis of the results of tests for activity.

Purification

Two different bacteriocins were isolated from the MRS culture of *L. acidophilus* LF221 by ammonium sulphate precipitation, cation-exchange chromatography, C_8 hydrophobic-interaction chromatography and reverse-phase FPLC chromatography. The results of purification are summarised in Table 3. Most of the bacteriocin activity (80%) was recovered during precipitation with ammonium sulphate. Application of the concentrate on an S-Sepharose column resulted in an additional 70% loss of the initial activity. During the chromatography on an octyl-Sepharose column, 80% of the applied activity was recovered. The highest losses of bacteriocin activity occurred during the last step in the purification, reverse-phase FPLC chromatography. Three successive runs were needed to isolate bacteriocins A and B. When the active fractions from the first chromatography on

Table 2 Effect of heat treatment and trypsin on the activity of a 10-fold concentrated bacteriocin preparation of *L. acidophilus* LF221. *AU* units of bacteriocin activity

Treatment	10^{-4} × Residual activity (AU ml^{-1})
Control (untreated sample)	5.1
Heat	
70 °C/10 min	5.1
70 °C/30 min	3.8
70 °C/60 min	2.6
100 °C/5 min	5.1
100 °C/10 min	3.8
100 °C/30 min	2.6
115 °C/15 min	0
Trypsin	
0.1 mg ml^{-1} (9.4 U ml^{-1})	5.1
1 mg ml^{-1} (94 U ml^{-1})	2.6
5 mg ml^{-1} (470 U ml^{-1})	0
Control (trypsin solution in buffer)	
0.1 mg ml^{-1} (9.4 U ml^{-1})	0
1 mg ml^{-1} (94 U ml^{-1})	0
5 mg ml^{-1} (470 U ml^{-1})	0

the reverse-phase column were applied again and eluted with a linear gradient ranging from 30% to 50% 2-propanol, two absorbance peaks were separated (Fig. 1). Two fractions that contained absorbance peaks and the most of bacteriocin activity were reapplied separately on the reverse-phase column and eluted with a linear gradient of 30%–40% 2-propanol. Elution of peptide A coincided with the absorbance peak at 32.5% 2-propanol and peptide B was eluted at the concentration of 34.2% 2-propanol. No increase in bacteriocin activity was observed when the fractions were mixed with peptides A and B.

Amino acid sequence

Two fractions, from the final steps of purification by FPLC reverse-phase chromatography, that contained peptides A and B respectively were analysed for N-terminal amino acid sequences. The N-terminal sequences of 46 amino acid residues of peptide A (5 of them unidentified) and of 35 amino acid residues of peptide B (2 of them unidentified) were determined. Unusual amino acids, such as lanthionin or methyllanthionin were not found in the two peptides (Fig. 2).

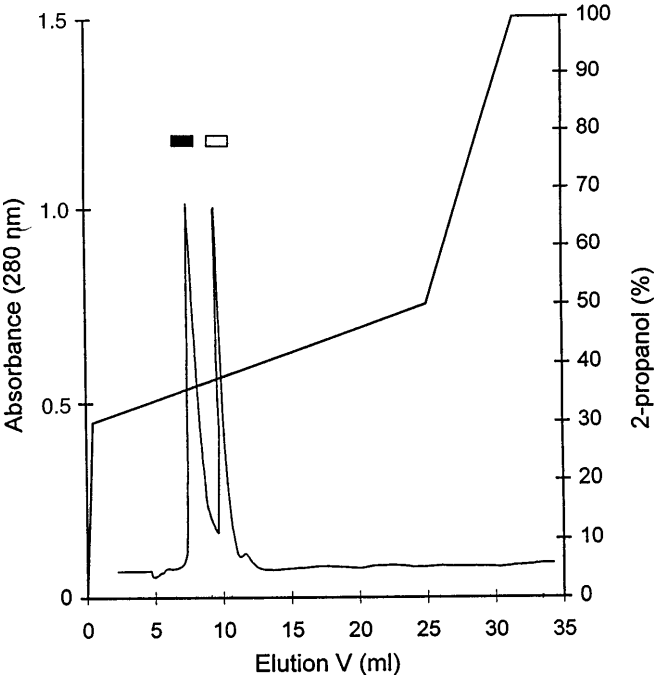

Fig. 1 C_2/C_{18} reverse-phase chromatography analysis of acidocins LF221 A and B. Two fractions with absorbance peaks contained most of bacteriocin activity and were further purified by FPLC. ■ Acidocin LF221 A, □ acidocin LF221 B

The sequenced parts of bacteriocin A and bacteriocin B contained 52% and 66% non-polar amino acid residues respectively, mainly glycine and alanine. The high content of glycine and other hydrophobic residues (valine, leucine, isoleucine) is characteristic of the II class bacteriocins of lactic acid bacteria. No significant homology was found between the amino acid sequences of bacteriocin A and the composition of other known bacteriocins of the lactic acid bacteria (NCBI database). The sequenced part of bacteriocin B contained 26% amino acids identical with those of brevicin 27.

Discussion

The main reason for isolating of bacteriocin(s) from the supernatant of the strain *L. acidophilus* LF221 was the very interesting spectrum of inhibitory activity of the strain, when the effects of pH and hydrogen peroxide were excluded. Two lactobacillus bacteriocins with an

Table 3 Purification of acidocins LF221 A and LF221 B of *L. acidophilus* LF221

Purification stage	Vol (ml)	Activity (AU ml^{-1})	Total activity (AU)	Recovery (%)
Culture supernatant	2000	125 000	250 000 000	100
Fraction				
I. Ammonium sulphate precipitation	400	500 000	200 000 000	80
II. S-sepharose column eluate	50	500 000	25 000 000	10
III. C_8 column eluate	10	2 000 000	20 000 000	8
IV A. FPLC fr. with acidocin LF221 A	1	50 000	50 000	0.020
IV B. FPLC fr. with acidocin LF221 B	1	150 000	150 000	0.060

A NNVNWGSVAG SXGKGAVMEI YFGNPILGXA

NGAATSLVLX QTAXXI

B (N)KWG(N)AVIGA ATGATRGVSW

X(A)(G)FGPWGMT AXG(L)(A)

Fig. 2 **A** NH_2-terminal amino acid sequence of acidocin LF221 A. **B** NH_2-terminal amino acid sequence of acidocin LF221 B. Amino acids that are not identified with certainty but are probable are shown in parentheses; *X* unidentified amino acid residue

extended spectrum of activity have been described so far: acidocin A, also inhibiting some food-borne pathogens including *Listeria monocytogenes*, and acidocin B, which inhibits *L. monocytogenes* and *Cl. sporogenes* (Kanatani et al. 1995; ten Brink et al. 1994). The spectrum of activity of bacteriocins of the strain LF221 was even wider, including a few genera that were not lactobacilli: *Lactococcus*, *Pediococcus*, *Staphylococcus*, *Enterococcus*, *Streptococcus, Listeria*, *Clostridium* and *Bacillus*. The activity against opportunistic *Cl. difficile,* a bacterium causing antibiotic-associated diarrhoea or even life-threatening pseudomembraneus colitis, and against other clostridia is of particular interest. Inhibition of *B. cereus* is also often desirable, but the strain LF221 was active only against particular strains of this species.

The biochemical properties of the two bacteriocins of LF221 were similar to those of the other lactobacillus bacteriocins, belonging to the group II lactic acid bacteria bacteriocins (<10 kDa, heat stability, sensitivity to proteinases, formation of aggregates). The results of SDS-PAGE electrophoresis did not show any evidence that two bacteriocins were present, probably because of their similar biochemical characteristics. While the activity was detected clearly after electrophoresis, no bands were visible at all after Coomassie blue staining, or one diffuse band was seen at the position of the inhibition zone. Similar observations were reported for sakacin B purification (Samelis et al. 1994), where staining with Coomassie blue failed to develop a band at the position where inhibition of the indicator lawn was detected, when crude sakacin B was applied on a gel. Also lactacin F could not be detected by Coomassie dye staining (Muriana and Klaenhammer 1991a). Another bacteriocin that could be detected on SDS-PAGE gel directly, but not by staining, was carnosin LA44 A (van Laack et al. 1992). The molecular mass of bacteriocins LF221 was estimated to be between 3500 Da and 5000 Da by SDS-PAGE electrophoresis. The final purification steps only showed that there were at least two bacteriocins in the supernatant fluid.

Ammonium sulphate precipitation of both bacteriocins of LF221 was quite successful, 80% recovery and 10% recovery after cation-exchange chromatography being comparable with results for the other lactobacillus bacteriocins. Ultrafiltration with a 10 kDa cut-off membrane or even 100 kDa could also be used to increase the concentration of the bacteriocins of LF221 since they were present in the culture supernatant in the form of aggregates. Practically all the activity was recovered after ultrafiltration. Great losses occurred during three repeated steps on a reverse-phase FPLC column, resulting in only 0.02% recovery of the initial activity of acidocin LF221 A and 0.06% of acidocin LF221 B. Some bacteriocins can lose their activity during purification and storage as a result of the activity of extracellular proteases. For natural and synthetic curvacin A and pediocin PA-1 it was shown that oxidation of methionine residues reduced bacteriocin titres (Fimland et al. 1996). As both bacteriocins of the strain LF221 contain at least one methionine residue, methionine oxidation could be an explanation of the high losses of activity. Molecular mass analysis would be necessary, as methionine oxidation results in an increased molecular mass of the oxidized form.

A number of bacteriocins of lactic acid bacteria, from class II, consist of two peptides, their activity depending on the complementary action of both components: plantaricin S, lactacin F, lactococcin G, lactococcin MN and bacteriocins of *L.plantarum* C11 (Nes et al.1996). Several attempts to detect an increased bacteriocin activity by mixing fractions containing bacteriocins A and B were not successful, indicating that there was no complementary activity between bacteriocins A and B.

The N-teminal amino acid composition of bacteriocins A and B was found to be different from those of the other bacteriocins, indicating that the two bacteriocins were novel. They were named acidocin LF221 A and acidocin LF221 B. As many bacteriocins from class II have similar N-terminal extensions, which are cleaved from the active bacteriocin during processing (Klaenhammer 1993), the characterization of bacteriocin(s) genes of the strain LF221 would be necessary for the comparision of bacteriocins.

As *L. acidophilus* LF221 is an intestinal isolate, which inhibits *Cl. difficile* and some other pathogenic bacteria, it may be interesting as a probiotic strain. The possibility of preventing *Cl. difficile* infections in patients treated with antibiotics with the help of strain LF221 should be investigated.

For all these reasons, mechanisms of action and genetic determinants of bacteriocins produced by the strain LF221 strain should be studied in detail. The genetic characterization of the genes involved in the production of both new bacteriocins is in progress.

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