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Antibacterial activity of plantaricin SIK-83, a bacteriocin produced by *Lactobacillus plantarum**

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Summary — *Lactobacillus plantarum* SIK-83 produces a bacteriocin, designated plantaricin SIK-83, which inhibits 66 of 68 lactic acid bacteria from the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*. A 500-fold dilution of *L. plantarum* SIK-83 MRS culture supernatant with phosphate buffer was sufficient to kill 10^5 cells/ml of *Pediococcus pentosaceus* within 120 s. The killing of a sensitive population followed exponential kinetics. It was shown that the bacteriocin binds specifically to sensitive cells but not to nonsensitive lactic acid bacteria, the producer strain or Gram-negative bacteria. Sensitive cells, after exposure to the bacteriocin, could be rescued by treatment with proteolytic enzymes. In buffer, plantaricin SIK-83 was adsorbed to the cell surface almost immediately, and morphological lesions were observed within 2 h after the cells were exposed to the bacteriocin. The lethal mode of action appeared to be due to damage to the cell membrane, resulting in cell lysis, which was detected by electron microscopy and by determination of released intracellular components.

lactic acid bacteria / bacteriocin / plantaricin / antagonism / fermentation

Introduction

Bacteriocins are antibacterial substances which are produced by many different bacterial species. Although the bacteriocins form a heterogenous group with respect to producing bacteria, antibacterial spectrum, mode of action, and chemical properties, they are by definition, according to Tagg *et al.* [1], protein-containing substances possessing bactericidal activity.

Among the lactic acid bacteria, bacteriocins have been described for *Lactobacillus* [2-6], *Pediococcus* [7,8] and *Streptococcus* [9-14]. The information regarding the kinetics and mechanisms of the lethal action of bacteriocins is primarily based on studies of colicins and bacteriocins produced by streptococci. A widely accepted hypothesis is that the mode of action occurs in 2 steps. In the first step, the bacteriocin is adsorbed to specific receptors on the cell surface and, after a time, the second step develops, which results in cell death. Bacteriocins differ in their lethal action and can initiate reactions which can inhibit energy production, syn-

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thesis of protein or nucleic acids, or alter membrane permeability and transport [1, 9, 12, 14–16]. It is of interest to determine whether bacteriocins from the lactobacilli act by similar or different mechanisms.

In a previous study [17] an antagonistic compound from *Lactobacillus plantarum* SIK-83 was shown to inhibit *Staphylococcus aureus,* but not Gram-negative bacteria. However, spheroplasts of Gram-negative bacteria were sensitive to the compound.

In this study, we show the antagonistic compound produced by *L. plantarum* SIK-83 to be a bacteriocin, designated plantaricin SIK-83, and present results with emphasis on: 1) the antibacterial spectrum; 2) killing kinetics; and 3) mode of action.

Materials and methods

Bacterial cultures

The producer of the bacteriocin has been classified as *L. plantarum* SIK-83 and was isolated from lactic acid-fermented carrots [18]. Other strains of lactic acid bacteria used are presented in Table I. The lactic acid bacteria were maintained and grown in MRS broth (Difco Labs, Detroit, MI). *Escherichia coli* (B-14) and *Pseudomonas fluorescens* (B-16) were obtained from the department culture collection (USDA-ARS) and cultivated in TSB (Difco).

Bacteriocin preparations

Lactobacillus plantarum SIK-83 was cultivated statically overnight (16 h) in MRS broth at 30°C. A cell-free bacteriocin solution was obtained by centrifuging the culture, followed by sterile filtration (0.22-μm filter) of the supernatant. This crude plantaricin SIK-83 was stable for at least 3 months at 22°C and used in the experiments, if not otherwise mentioned.

A concentrated and partially purified plantaricin SIK-83 was prepared by cultivation of *L. plantarum* SIK-83 overnight at 37°C in a defined medium, described by McFeeters and Chen [19], supplemented with 2% glucose and 1% MRS broth. The cells were removed by centrifugation, and then the supernatant was concentrated 50 × by ultrafiltration using a membrane with a molecular weight cutoff of 10,000 Da. The ultrafiltered bacteriocin was dialyzed against cold buffer (50 mM K_2PO_4, 0.1 mM EDTA, pH 7.8), filter-sterilized and used in the mode of action studies as a partially purified preparation.

Assay of bacteriocin activity

Plantaricin SIK-83 was checked for activity using an MRS agar well diffusion method based on the assay system described by Tagg and McGiven [20]. After diffusion of 10–100 μl of sample, the plates were overlaid with 8 ml of an MRS soft agar (0.8%) containing a sensitive indicator culture (10^6 CFU/ml). After incubation for 16–24 h at 30°C, clear zones around wells indicated bacteriocin activity.

Bacterial counts

0.1-ml samples from appropriate dilutions were placed on MRS agar according to the spread-plate method. The colonies were counted after 2 days of incubation at 30°C, and the bacterial numbers were expressed as CFU/ml.

Antagonistic spectrum

The inhibitory spectrum of *L. plantarum* SIK-83 was determined by using the agar spot-test method. Two μl of an overnight culture was spotted on an MRS agar plate and incubated for 16 h at 30°C. The plates were then overlaid with MRS soft agar containing an indicator culture. After incubation for 24 h at 30°C, the indicator was considered to be inhibited if the *L. plantarum* SIK-83 colony was surrounded by a clear zone.

Killing kinetics

The loss of viability of a population of sensitive cells was determined in MRS broth and in phosphate buffer (50 mM; pH 6.5) respectively, as a function of time and amount of plantaricin SIK-83 added. The indicator bacteria were added as a log phase culture to a final concentration of $\approx 5 \times 10^5$ CFU/ml. Plantaricin SIK-83 was added in amounts giving a final concentration of the bacteriocin ranging from 0.05–26% (v/v, *i.e.*, 2,000- to 4-fold dilution, respectively). Samples were withdrawn at regular times and viable counts were performed. Killing rates were calculated from the slope of survivor curves for different bacteriocin concentrations.

Adsorption of plantaricin SIK-83 to bacterial cells

The bacteriocin was examined for adsorption to both sensitive and nonsensitive cells. Cells from overnight cultures were harvested by centrifugation, washed twice in 50 mM phosphate buffer, pH 6.5, and resuspended in buffer to a final concentration of 10^8–10^9 CFU/ml. Four hundred μl of bacteriocin preparation was then added to 2 ml of cell suspension. After incubation for 30 min at 22°C, the cells were removed by centrifugation at 15,000 × *g* for 10 min, and the supernatant was checked for bacteriocin activity using the agar well method.

Adsorption of the bacteriocin to heat-killed (autoclaved at 121°C for 15 min) or protease-treated cells was also examined. Protease treatments were carried out by adding 400 μl of protease (Sigma

Table I. Lactic acid bacteria and their sensitivity to bacteriocin from *L. plantarum* SIK-83.

Indicator	Exptl. no.	Origin[a]	Sensitivity
Lactobacillus ssp.			
L. acidophilus	LA 28	FFL	−
L. brevis	LA 25	FFL FBB-50	+
	LA 26	FFL FBB-70	+
	LA 36	ATCC 14869;NRRL B-4527	+
	C 136	FFL	+
	28	FFL	+
	103	FFL	+
L. buchneri	LA 30	ATCC 4055;NRRL B-1837	+
L. casei	LA 37	NRRL B-1445	+
L. casei ssp. *pseudoplantarum*	LA 38	NRRL B-4560	−
L. caucancus	LA 80	NRRL B-1839	+
L. cellobiosis	LA 31	ATCC 11739;NRRL B-1840	+
	LA 32	NRRL B-1915	+
L. coryneformis ssp. *torqueus*	LA 41	NRRL B-4390	+
L. coryneformis ssp. *coryneformis*	LA 42	NRRL B-4391	+
L. fermentum	LA 35	ATCC 14931;NRRL B-4524	+
L. hilgardi	LA 33	ATCC 8290;NRRL B-1843	+
L. plantarum	LA 23	FFL WSO	+
	LA 24	FFL FBB-442	+
	LA 51	NCDO 965	+
	LA 70	ATCC 14917	+
	LA 87	NCDO 15	+
	LA 88	NCDO 16	+
	LA 89	NCDO 82	+
	LA 90	NCDO 340	+
	LA 91	NCDO 341	+
	LA 92	NCDO 343	+
	LA 93	NCDO 352	+
	LA 94	NCDO 354	+
	LA 95	NCDO 363	+
	LA 96	NCDO 1193	+
	LA 97	NCDO 1194	+
	LA 98	NCDO 1752	+
	LA 99	NCDO 1939	+
	304+	FFL	+
	304-	FFL	+
	C11	FFL	+
	SIK-83	SIK	−
	MOP-3	FFL	+

L. salivarius			
ssp. *salivarius*	LA 39	NRRL B-1949	+
ssp. *salicinius*	LA 40	NRRL B-1950	+
L. viridescens	LA 34	ATCC 12706;NRRL B-1951	+
L. xylosus	LA 43	NRRL B-4449	+
Lactobacillus sp.	LA 67	FFL	+
	LA 68	NCIB 9430	+
	LA 69	NCIB 9431	+
Leuconostoc ssp.			
L. dextranicum	LA 7	ATCCC 19255;NRRL B-3469	+
L. lactis	LA 6	ATCC 19256;NRRL B-3468	+
L. mesenteroides	LA 9	FFL	+
	LA 10	FFL LC 33	+
	LA 11	FFL LM 42	+
	LA 81	ATCC 8293	+
L. paramesenteroides	LA 8	NRRL B-3471	+
Pediococcus ssp.			
P. acidilactici	LA 74	ATCC 33314	+
	LA 82	NRRL B-5627	+
P. cerevisiae	LA 2	FFL FBB 39	+
	LA 63	FFL SP 3	+
	LA 64	FFL 12	+
	LA 65	FFL 1	+
P. dextranicus	LA 77	ATCC 33087	+
P. pentosaceus	LA 61	FFL L-7230	+
	LA 72	NRRL B-11465	+
	LA 73	ATCC 25745	+
Pediococcus sp.	LA 4	Microlife AFERM 772	+
Streptococcus ssp.			
S. cremoris	LA 14	ATCC 9625;NRRL B-634	+
S. faecalis	LA 71	ATCC 14508	+
	LA 85	JH2-2, D. Clewel, Univ. Michigan	+
S. lactis	LA 78	LM 2302, L. McKay, Univ. Minnesota	+
S. sanguis	LA 79	ATCC 35105	+

[a] *Abbreviations:* **ATCC**, American Type Culture Collection (Rockville, MD, USA); **NRRL**, Northern Regional Research Laboratory, USDA (Peoria, IL, USA); **NCDO**, National Collection of Dairy Organisms (Reading, UK); **SIK**, The Swedish Food Institute (Goteborg, Sweden); **NCIB**, National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland); **Microlife**, Microlife Technics (Sarasota, FL, USA); **FFL**, US Food Fermentation Laboratory, USDA-ARS (Raleigh, NC, USA).

Table II. Plantaricin SIK-83 adsorption to heat- and protease-treated, sensitive and nonsensitive bacteria.

Strain	Sensitivity	Bacteriocin adsorption		
		Living cells	Heat-killed cells	Protease-treated cells
Lactobacillus acidophilus (LA 28)	−	−	−	−
Lactobacillus casei ssp. *pseudoplantarum* (LA 38)	−	−	−	−
Lactobacillus plantarum (LA 95)	+	+	+	+
Lactobacillus plantarum (SIK 83)	−	−	+	−
Lactobacillus salivarius ssp. *salicinius* (LA 40)	+	+	+	+
Leuconostoc mesenteroides (LA 81)	+	+	+	+
Pediococcus pentosaceus (LA 61)	+	+	+	+
Streptococcus lactis (LA 78)	+	+	+	+
Escherichia coli (B 16)	−	−	−	−
Pseudomonas fluorescens (B 14)	−	−	−	−

Chemical Co., St. Louis, MO; 10 mg/ml) to 2 ml of a cell suspension. After incubation for 1 h at 22°C, the cells were washed in 50 mM phosphate buffer and resuspended in 2 ml of the same buffer. Four hundred μl of the plantaricin SIK-83 was added separately to heated or protease-treated cells, and adsorption of the bacteriocin was determined as described above.

Protease rescue

To determine if a proteolytic enzyme treatment affects the killing of the cells by plantaricin SIK-83, *Pediococcus pentosaceus* (LA-61) was treated with protease (10 mg/ml). The amount of protease used totally inactivated plantaricin SIK-83. The bacteria were washed twice in sterile phosphate buffer (50 mM; pH 6.5), and a diluted cell solution containing ≈ 500 CFU was transferred to a sterile vacuum filter (Nalgene, Nalge Co., Rochester, NY). The cells were then exposed to 2 ml of plantaricin SIK-83 for 2 min and to protease for 30 min in different exposure sequences (Table III). Between all treatments, the cells were washed in 10 ml of the buffer. After the treatments performed at 20°C, tempered MRS agar was added to the filter and the CFU numbers were counted after incubation for 2 days at 30°C.

Spectrophotometric determination of cell lysis

Increases in UV-absorbent material when sensitive cells were lysed due to plantaricin SIK-83 activity were measured at 260 nm. Log phase cells of *L. plantarum* (LA-95) were washed twice in sterile saline solution (0.85% NaCl) and resuspended in saline to a cell concentration of $\approx 2-4 \times 10^8$ CFU/ml. Partially purified plantaricin SIK-83 was added to the cell suspension, giving a final bacteriocin concentration of 10% (v/v). The absorbance was determined during incubation for up to 8 h. Decreases in optical density were determined simultaneously at a wavelength of 650 nm.

Electron microscopy

The lethal mode of action of plantaricin SIK-83 on cells was also studied using transmission electron microscopy. Log phase cells of *L. plantarum* (LA-95), *Leuconostoc mesenteroides* (LA-81) and *P. pentosaceus* (LA-61) were washed twice in sterile 50 mM phosphate buffer, pH 6.5, and then resuspended in buffer to a cell concentration of $\approx 2 \times 10^8$ CFU/ml. One hundred μl of partially purified bacteriocin was added per ml of cell suspension and incubated at 37°C. One-ml samples were withdrawn at sequential time intervals, cells were spun down and resuspended in 1 ml of McDowel and Trump's [21] fixative. Preparation of samples for electron microscopy was carried out according to Wiegel and Dykstra [22].

Table III. The effect of protease treatments on the bacteriocin sensitivity of *Pediococcus pentosaceus*.

Treatment*	CFU number
Cells	400
Cells + bacteriocin	0
Cells + bacteriocin + protease	500
Cells + protease + bacteriocin	4
Cells + protease	500

*In sequence of addition.

Results and Discussion

Characteristics of the bacteriocin

Lactobacillus plantarum SIK-83 produced an extracellular antibacterial substance which was sensitive to proteolytic enzymes [17] and bactericidal in mode of action (Fig. 1). According to the definition by Tagg *et al.* [1], the compound could be classified as a bacteriocin and was designated as plantaricin SIK-83. The crude MRS preparation of plantaricin SIK-83 was heat-resistant and could be boiled for 2 h or autoclaved at 121°C for 15 min and remain active (data not shown). In a previous study [17], it was reported that the antagonistic compound lost its effect after heat treatment at 121°C for 15 min. However, in that study the preparation had been ultrafiltered and dialyzed, and the heat treatment carried out at different pH and under different ionic strength conditions.

Antibacterial spectrum

Previously, *L. plantarum* SIK-83 was shown to inhibit *S. aureus*, but not Gram-negative bacteria [17]. In this study, 68 different lactic acid bacteria from the genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* were tested. Sixty-six strains were inhibited by plantaricin SIK-83 (Table I). Thus, this bacteriocin possessed an extended activity spectrum on Gram-positive bacteria, which has also been reported for other bacteriocins from lactic acid bacteria [7-10, 13]. In contrast, other bacteriocins are characterized by a narrow spectrum of inhibitory activity against closely related species [2, 3, 5, 6, 9-11].

The production of a bacteriocin with a broad spectrum of antibacterial activity against other genera of lactic acid bacteria can be an important property for starter cultures and is of special interest in controlled lactic acid fermentation of plant materials such as vegetables and silage, which naturally contain competing lactic acid bacterial flora. In vegetable fermentations, starter cultures are only used to a limited extent commercially because it is difficult to achieve a pure culture fermentation with an added starter due to competition from naturally occurring lactic acid bacteria on the vegetables [23].

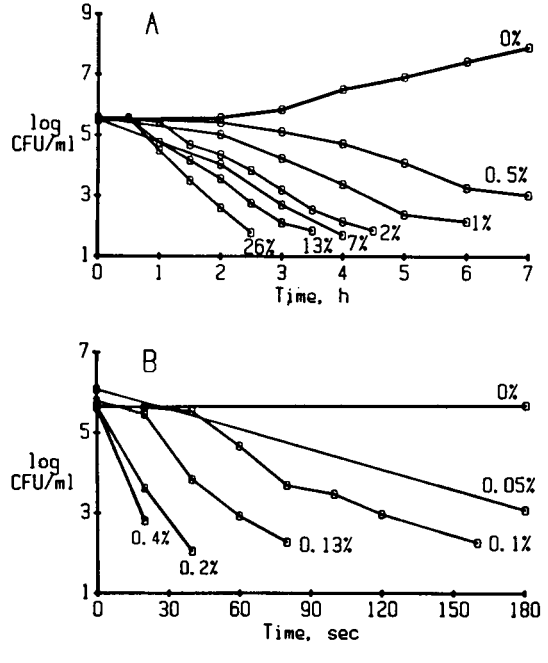

Fig. 1. Survivor plots of *P. pentosaceus* as a function of time at different concentrations of plantaricin SIK-83 in MRS broth (A) and in 50 mM phosphate buffer, pH 6.5 (B).

Killing kinetics

Growth of *L. plantarum* SIK-83 in MRS broth resulted in high bacteriocin production; the culture supernatant could be diluted 1,000-fold in buffer and kill 5×10^5 cells/ml within 3 min (Fig. 1). The killing effect of plantaricin SIK-83 was more pronounced when the test organism was suspended in phosphate buffer than in MRS broth (Fig. 1). This effect could be a result of the presence of interfering substances in the MRS broth. Survivor plots of a sensitive population by plantaricin SIK-83 suggested first-order kinetics (Fig. 1). This was further confirmed in Figure 2, where a linear correlation was found when kill rates were plotted *versus* plantaricin SIK-83 concentration. The graphs in Figure 3 show that within a certain range there was a linear correlation between plantaricin SIK-83 concentration and the time it took to reduce the CFU number of a sensitive population to 1% of the initial CFU number. However, at higher bacteriocin concentrations the time decrease was comparatively slower. This was probably due to the bacteriocin being in excess in relation to cell number, so that no further bacteriocin could be adsorbed to the cell surfaces. Due to the lack of a pure bacterio-

cin, it was difficult to tell whether it was a single-hit or a multi-hit type kinetic. Little information is available concerning the kinetics of killing of bacteriocins from lactic acid bacteria.

Adsorption

It was shown that plantaricin SIK-83 bound specifically to sensitive cells, but not to non-sensitive lactic acid bacteria or Gram-negative bacteria (Table II). This indicated that the attachment of the bacteriocin occurred at specific receptors, resulting in lethal action. The fact that the bacteriocin binding to cells was not affected by either heat-treatment or protease-treatment of the cells (Table II) indicated that the receptors on the cell surface may not be of protein nature. This can also be concluded from Table III, where it is shown that protease-treated cells were sensitive to the bacteriocin. Plantaricin SIK-83 did not bind to the producer strain either, and the binding was not affected by protease treatment of the cells. However, heat-killed (autoclaved) cells of *L. plantarum* SIK-83 bound the bacteriocin, indicating that immunity to the bacteriocin might involve heat-sensitive components that block adsorption of the bacteriocin (Table II). The difference in resistance and binding between Gram-positive and Gram-negative bacteria, which has also been reported by others [8, 10, 13], may be due to the absence or presence of specific receptors for the bacteriocin molecule. The lethal effect was initiated by the rapid attachment of the bacteriocin to the cell surface (Table III and Fig. 1). However, bacteriocin binding did not necessarily result in cell death. When the bacteriocin was bound to a sensitive cell, the addition of protease could rescue the cells (Table III). This could be a result of either inactivation, or release of the bacteriocin from the cells or both. The phenomenon is known as protease-rescue and has been shown in colicins [16]. Since plantaricin SIK-83 binding did not appear to be of a protein nature, the rescue seemed to be the result of inactivation of the attached bacteriocin rather than release of bacteriocin from the cells.

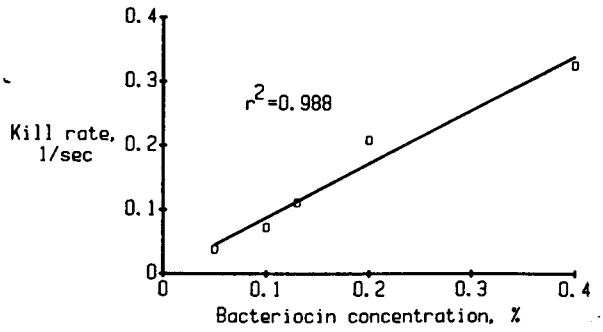

Fig. 2. Killing rates of *P. pentosaceus* as a function of plantaricin SIK-83 concentrations in 50 mM phosphate buffer, pH 6.5.

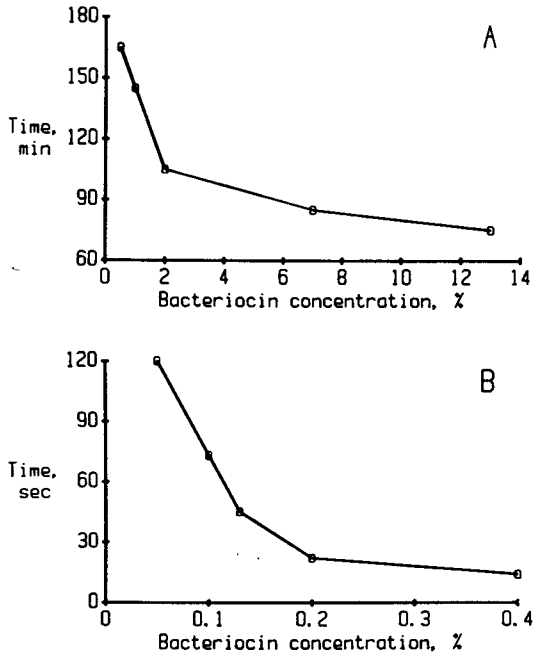

Fig. 3. Time to reduce the CFU numbers of *P. pentosaceus* with 99% or 2 log units as a function of plantaricin SIK-83 concentration in MRS broth (**A**) and in 50 mM phosphate buffer, pH 6.5 (**B**).

Mode of action

The rapid lethal effect noticed when *P. pentosaceus* was exposed to plantaricin SIK-83 in phosphate buffer (Fig. 1) was not the actual time it took to kill the cells, but rather the time during which the bacteriocin was adsorbed to the cells. Examination of cells under light microscope showed that morphological changes could be seen within 2 h after exposure to the bacteriocin. These effects were even more obvious by transmission electron microscopy and are presented in Figure 4. The difference in time between attachment of plantaricin SIK-83 and microscopically detected cell lysis probably represented the time required for penetration of the bacteriocin into the cell envelope, where

Fig. 4. Electron micrographs of *L. plantarum* exposed to plantaricin SIK-83 for 0 h (**a**), 1.5 h (**b**), 2.5 h (**c**), and 3.5 h (**d**). (**e**) shows a detail of (**c**). Scale bars correspond to 1 μm.

Table IV. Absorbance changes in cell cultures (*L. plantarum* LA-95) containing plantaricin SIK-83.

Incubation time (h)	Absorbance				Changes in absorbance of plantaricin SIK-83 treated cells relative to control	
	Cells in saline (with 10% plantaricin SIK-83)		Cells in saline (control)			
	650 nm	260 nm	650 nm	260 nm	ΔA_{650}	ΔA_{260}
0	0.180	0.00	0.180	0.00	0.00	0.00
4	0.170	0.42	0.180	0.09	−0.10	0.39
8	0.130	1.05	0.185	0.22	−0.55	0.83

*Partially purified preparation (see Materials and methods).

the lethal effects appeared to occur. As can be seen in Figure 4, the cell membrane appeared to be damaged, resulting in cell lysis. Identical results have been obtained for *L. plantarum, L. mesenteroides* and *P. pentosaceus*. The leakage of intracellular proteins and nucleic acids, as indicated by increases in absorbance at 260 nm, was correlated to cell lysis (Table IV). Little data is available concerning the mode of action for bacteriocins from lactic acid bacteria, but bacteriocins from *Streptococcus* have been shown to inhibit macromolecular synthesis and alter membrane permeability [12, 14, 24]. The lethal effect of plantaricin SIK-83 might be due to direct enzymatic activity or to the activation of the lytic enzyme system.

Conclusion

Our results show that *L. plantarum* SIK-83 produces a bacteriocin with a broad of spectrum activity against lactic acid bacteria. The lethal mode of action takes place in 2 steps: a comparatively rapid attachment of the bacteriocin to the cell surface, followed by killing of the cells and cell lysis. The killing of a sensitive population follows exponential kinetics, and the bacteriocin acts, directly or indirectly, by damaging the cell membrane.

Note
Recent experiments have shown that *L. plantarum* SIK-83 might be *Streptococcus lactis* and that plantaricin is similar to nisin.

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