RESEARCH ARTICLE

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Bacterial membrane injuries induced by lactacin F and nisin

Abstract The combined action of nisin and lactacin F, two bacteriocins produced by lactic acid bacteria, is additive. In this report, the basis of this effect is examined. Channels formed by lactacin F were studied by experiments using planar lipid bilayers, and bactericidal effects were analyzed by flow cytometry. Lactacin F produced pores with a conductance of 1 ns in black lipid bilayers in 1 mM KCl at 10 mV at 20 °C. Pore formation was strongly dependent on voltage. Although lactacin F formed pores at very low potential (10 mV), the dependence was exponential above 40 mV. The injuries induced by nisin and lactacin F in the membranes of *Lactobacillus helveticus* produced different flow cytometric profiles. Probably, when both bacteriocins are present, each acts separately; their cooperation may be due to an increase in the number of single membrane injuries.

Keywords Lactacin F · Nisin · Lactic acid bacteria (LAB) · Planar lipid bilayer · Flow cytometry

Introduction

A large variety of antibiotics and other antimicrobial agents have been used not only to combat infections but also for other applications, for example to promote growth in livestock. This has resulted in the emergence of antibacterial drug resistance, which has led to major problems in the control of infectious diseases. Many strategies have been used to find new antimicrobial agents, including research on short peptides produced by bacteria. Bacteriocins produced by lactic acid bacteria (LAB-bacteriocins) are of interest because of their effect on organisms responsible for both infectious diseases and food spoilage. have been explored and occasionally used as food preservatives [18]. Use of the LAB-bacteriocin nisin is allowed in most industrialized countries [15]; however, its use as a preservative is limited due to both its narrow spectrum and the high frequency of mutations leading to nisin-resistance. In a previous report we explored the antibacterial action of LAB-bacteriocins pairs [25]. Several combinations were described as having a synergistic or additive effect, whereas, in some cases, indifference and even antagonism were detected. Nisin and lactacin F have additive bactericidal activity. Nisin is a well-known LAB-bacteriocin whose mechanism of action has been extensively investigated [24, 32, 34]. Lactacin F is produced by *Lactobacillus johnsonii* [19] and has antibiotic activity against other species of *Lactobacillus* and against *Enterococcus faecalis* [1]. It is composed of two polypeptide subunits, LafA and LafX, which are encoded together with a promoter and the putative immunity protein ORFZ on a 1-kb polycistronic operon, similar to the genetic organization of lactococcin M [2,14]. LafA and LafX are produced with N-terminal extensions characterized by a special cleavage site [3]. Both subunits are necessary for biological activity, i.e. the two have to be expressed together [14]. The action of lactacin F on other species of *Lactobacillus* has been investigated in detail [1]. Adding lactacin F to the susceptible cells results in a sudden loss of internal potassium and rapid depolarization of the cytoplasmic membrane. The response to uncouplers of oxidative phosphorylation suggests that the proton-motive force is not essential for lactacin F action on target cells. This is in contrast to the effect of other bacteriocidins, such as lantibiotics, in which membrane potential plays an essential role [21,29].

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Traditionally the criterion of whether a bacterium is alive has been its ability to originate colonies on appropriate media. However, when spoilage has to be prevented this criterion is irrelevant and should be revised. In the last few years, new concepts have emerged, e.g. dormant bacteria, stressed bacteria, or viable but non-culturable (VBNC) bacteria. Most of these define bacterial populations whose individuals are able to metabolize, and spoil foods, but cannot be cultured in the laboratory [5,10]. Flow cytometry is used to study injured bacteria because it allows membrane integrity to be assessed. Accordingly, bacteria can be classified into four groups: (1) dead bacteria, in which the membrane integrity is severely compromised and staining is indiscriminate; (2) intact cells, without alterations in their membranes, which have selective permeability for supravital stains and completely exclude others; (3) metabolically active bacteria, which retain the ability to convert substrates, conserve some membrane potential, accumulate cationic stains and show active extrusion (efflux), but cannot form colonies; and (4) viable bacteria, able to divide and to form colonies. In order to interpret the basis of their cooperative effect, we used flow cytometry to estimate the extent of bacterial death induced by lactacin F and nisin, separately and in combination. Lactacin F forms transient channels in artificial membranes at low transmembrane potential [1]. These channels are anion-selective, like those formed by nisin and Pep 5, probably because LafA and LafX are cationic peptides like certain defensins. Nevertheless, lactacin F showed a strong, almost symmetric voltage-dependence, which resulted in a dramatic increase in membrane conductance above 50 mV. These results suggest that lactacin-F channel formation is similar to that of other cationic peptides, such as alamethicin [16], mellitin [33], and the lantibiotics [9,29]. Here we compare the effects of lactacin F on the cytoplasmic membrane of *Lactobacillus helveticus* and on planar bilayer membranes.

Here we describe the effect of lactacin F on lipid bilayer membranes. Furthermore, flow cytometry, a powerful tool in bacteriology [4], was used to study the antibacterial effect of lactacin F and nisin separately and in combination.

Materials and methods

Bacterial strains

Lactacin F was obtained from *Lactobacillus johnsonii* VPI 11088. The indicator strains are listed in Table 1. Brain heart infusion (BHI), tryptone soy(TS), and De Man Rogosa Sharp (MRS) media were purchased from Difco (Detroit, Mich., USA). Culture conditions are indicated in Table 1.

LAB-bacteriocins

Nisin was purchased from Sigma (St. Louis, Mo., USA). Lactacin F was obtained and purified as follows: 1 l of supernatant of a culture of one of the producer strains was maintained at 8 °C with continuous stirring. Ammonium sulfate was added to 30% and the liquid was stored at 8 °C for 18 h and then centrifuged at 12,000 g for 15 min at 6 °C. The floating film was harvested and centrifuged again. The resulting film was suspended in 50 mM phosphate buffer, pH 7. Protein was electrophoresed in a SDS-polyacrylamide gel [22]. The thick band corresponding to lactacin F was cut out and suspended in 800 µl phosphate buffer with 0.1% SDS, stored for 3 days at 4 °C and centrifuged (10,000 g for 10 min). The supernatant was harvested and stored at –80 °C after titration by critical dilution on solid medium.

Analytical procedures

Protein was measured by the method of Bradford [11]. Lactacin F activity is expressed in arbitrary units/ml after measurement by the critical dilution method on solid media. Briefly, MRS agar plates were covered with 5 ml MRS soft agar seeded with 10^6 cfu/ml of indicator strain (*L. helveticus*). Wells of 8 mm diameter were made and filled with 100 µl of the lactacin at appropriate dilutions; the plates were then incubated. Arbitrary units were defined as the reciprocal of the highest dilution that gave rise to a discernible inhibition zone.

Experiments with black lipid membranes

Black-lipid bilayer membranes were formed from a 1% solution of a variety of lipids (Avanti Polar Lipids, Alabaster, Ala., USA) in *n*-decane as described previously [7]. The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of about 0.5 mm^2, across which the membranes were formed. The aqueous salt solutions (Merck, Darmstadt, Germany) were used unbuffered and had a pH of around 6. Lactacin F was added from concentrated stock

Table 1. Origin and culture conditions of the lactic acid bacteria assayed

Bacterial strain	Source	Culture conditions
Listeria monocytogenes 1	DRCCRF[a] Montpellier (Dr. N. Richard)	BHI, 16 h at 37 °C, aerobic
Listeria monocytogenes 13	DRCCRF[a] Montpellier (Dr. N. Richard)	BHI, 16 h at 37 °C, aerobic
Listeria innocua 11	DRCCRF[a] Montpellier (Dr. N. Richard)	BHI, 16 h at 37 °C, aerobic
Listeria ivanovii 7	DRCCRF[a] Montpellier (Dr. N. Richard)	BHI, 16 h at 37 °C, aerobic
Listeria welsheri 4	DRCCRF[a] Montpellier (Dr. N. Richard)	BHI, 16 h at 37 °C, aerobic
Lactobacillus helveticus CIP 76.19	CIP	MRS, 18 h at 37 °C, 5–10% CO_2
Lactobacillus fermentum CIP 102980	CIP	MRS, 16 h at 37 °C, 5–10% CO_2
Pediococcus pentosaceus	LBCM[b]	MRS, 16 h at 37 °C, 5–10% CO_2
Staphylococcus aureus 7	LBCM[b]	TS, 16 h at 37 °C, aerobic
Enterococcus faecalis ATCC 10541	ATCC	TS, 16 h at 37 °C, aerobic

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solutions to the aqueous phase on one side, bathing a membrane in the black state. The temperature was kept at 25 °C throughout. The membrane current was measured with a pair of Ag/AgCl electrodes with salt bridges switched in series with a voltage source and a current amplifier (Keithley 427). The output signal of the amplifier was monitored on a strip-chart recorder and was fed via an A/D converter into a personal computer. The digitized data were analyzed with a home-made computer program. Zero-current membrane potential was measured by establishing a salt gradient across membranes containing 100–1,000 lactacin F channels as described elsewhere [8].

Flow cytometry

Lactobacillus helveticus were grown at 37 °C in MRS until an OD_{600} of 1.5, and then used as susceptible alive intact bacteria. Previous experiments established the optimal culture conditions to minimize artifacts due to dead and/or injured bacteria. Bacteria were resuspended in 1 ml phosphate buffer at 10^6 bacteria/ml (100% alive). An identical bacterial suspension was killed by incubating it at 60 °C for 15 min (100% injured bacteria). In order to prepare a standard of viability, different percentages of dead/ alive bacteria were mixed and stained with the Baclight kit (Molecular Probes, Leiden, Netherlands) for exactly 10 min. Baclight is a mixture of syto 9, a green fluorescent nucleic-acid stain which is membrane permeant, and propidium iodide (PI), a red fluorescent nucleic acid stain that penetrates only bacteria with damaged membranes. Samples were analyzed in a flow cytometer FACSCalibur (Becton Dickinson), and fluorescence was detected at 525 nm (syto 9) and 635 nm (PI); alive cells were syto+/PI–, while dead cells were syto–/PI+. For each sample 10,000 individual bacteria were recorded. Experiments with de-energized bacteria were performed by using 100 μM *m*-chloro-phenylhydrazone-carbonyl-cyanide (CCCP) 5 minbefore adding the LAB-bacteriocins.

Results and discussion

Purification of lactacin F

Lactacin F purified by the method described elsewhere [26] was not suitable for use in planar lipid bilayer experiments. We thus re-purified it by electrophoresis in SDS-PAGE, cutting out the thick band, and subsequently eluting the band. The purity of these preparations was tested by fast protein liquid chromatography (FPLC): a single peak was observed on the chromatograms (data not shown) corresponding to the active fraction. The lactacin F obtained in this way was pure enough to ensure that all pores were formed by lactacin F.

Interaction of lactacin F with lipid bilayer membranes

The addition of lactacin F leads to a rapid decrease in the membrane potential of *Enterococcus faecalis*, accompanied by a loss of intracellular K^+ [1]. It has been suggested that these effects are the result of channel formation in the cytoplasmic membrane by the two components of lactacin F, the peptides LafA and LafX that form the lactacin F complex [19]. To test this hypothesis, lipid bilayer experiments were carried out. Lactacin F was added in small concentrations (20 μg/

ml) to the aqueous phase of one or both sides of a black lipid membrane made of diphytanoyl phosphatidylcholine (DPhPC)/*n*-decane. After a lag time of about 2 min, the membrane conductance started to increase and reached a conductance maximum after about 20–30 min (Fig. 1, filled circles).

Furthermore, a considerable increase of the current noise of the membrane indicating rapid fluctuations of the conductive units was observed. The effect of lactacin F on the conductance of lipid bilayer membranes was strongly dependent on its concentration in the aqueous phase. When the lactacin F concentration was decreased by a factor of two (addition of 10 μg/ml to one or both sides of the membrane), the conductance increase was considerably smaller (see filled squares in Fig. 1). A possible explanation for this is that several lactacin F molecules are needed to form a conductive unit. In these experiments, several different lipids were tested to check whether the effects described above were lipid-specific. There was no evidence of lipid specificity, even when the membranes were formed from negatively charged phosphatidylserine. Therefore, most experiments were done with the neutral lipid DPhPC, which has branched side chains.

To study the putative conductive units in more detail, smaller amounts of lactacin F were added to black lipid membranes and the sensitivity of the current measuring device was increased. Figure 2 shows a current record of a DPhPC membrane under these conditions.

About 1 minbefore starting the record, lactacin F was added in a concentration of 5 μg/ml with stirring to allow equilibration to the *cis*-side of the membrane. About 2 minafter starting the record, the current trace
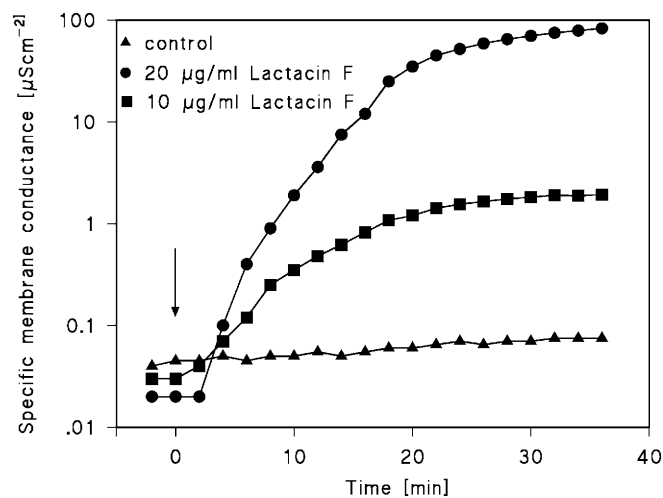

Fig. 1. Increase in current as a function of time after adding 20 μg/ ml lactacin F to both sides of a black diphytanoyl phosphatidylcholine (DPhPC)/*n*-decane membrane bathed in 1 M KCl (*filled circles*). *Filled squares* represent another experiment in which 10 μg lactacin F/ml was added to both sides of the membrane. *Filled triangles* represent a control experiment in which no lactacin F was added to the aqueous phase. The applied voltage was 20 mV; T = 20 °C. Lactacin F was added 10 min (corresponding to the *arrow*) after the membrane had been in the black state
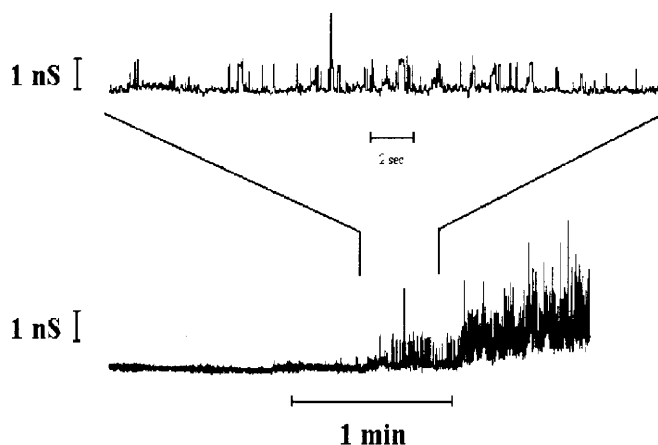
Fig. 2. Single-channel recording of a DPhPC/*n*-decane membrane in the presence of 1 μg lactacin F/ml added to the *cis*-side of the membrane. The aqueous phase contained 1 M KCl. The membrane potential applied at the *cis*-side was 10 mV; T = 20 °C. The time scale of part of the single-channel conductance recording was spread by a factor of five to allow resolution of the single channels

Fig. 3. Histogram of the probability *P*(G) of the occurrence of a given conductivity unit observed with DPhPC/*n*-decane membranes in the presence of 1 μg lactacin F/ml. *P(G)* is the probability that a given conductance increment *G* is observed in single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The membrane potential applied was 10 mV; T = 20 °C. The average single-channel conductance was 1 ns for 155 single-channel events (left-hand side maximum) and 2.9 ns for 48 events (right-hand side maximum). Note the very broad distribution of the channels

became noisy and showed spikes. When the time resolution of the record was drastically increased (see inset in Fig. 2), the spikes could be identified as membrane channels with a single-channel conductance of 1 ns which had average open times of about 20 ms. When the record was continued, higher conductance channels occurred. These had on average a single-channel conductance of about 3 ns but a lifetime of 5 ms at a membrane potential of about 10 mV (as in Fig. 2). For much longer recordings, the current trace became so noisy that it was impossible to resolve single-channels even at the increased time resolution. When the concentration of lactacin F was decreased to 1 μg/ml, channels in single-channel recordings were only occasionally observed, which indicated that the lactacin F concentration was below the critical limit needed for the formation of conductive units.

Figure 3 shows a histogram of the single-channel distribution at 10 mV obtained from recordings similar to thoseshown in Fig. 2. However, as was already visible from the data in Fig. 2, there were two maxima for the lactacin F channels, one was centered around 1 ns and the other around 3 ns. In addition to KCl, 1 M LiCl and 1 M K-acetate were used in single-channel experiments.

Although the single-channel distributions had the same width (Fig. 3) it was possible to deduce some anion selectivity from the single-channel data because conductance was 1 ns in 1 M KCl, 0.9 in 1 M LiCl and 0.65 in K-acetate. Similar results were observed for the channel that had a conductance of about 3 ns in 1 M KCl. In single-channel and in multi-channel experiments, we also measured the conductance as a function of salt concentration in the aqueous phase. Whereas the conductance curve vs lactacin F concentration was very steep (see above), linear increments of conductance vs salt concentration were determined, which indicated that the salt concentration had only a small influence, if any, on the formation of the conductive units.
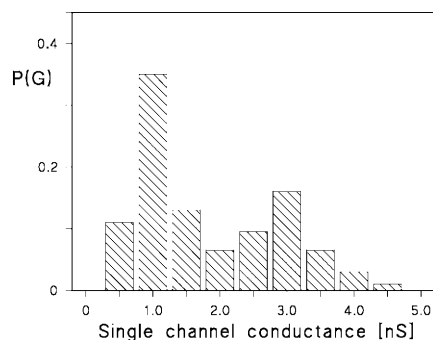
Voltage-dependence of the lactacin-F-induced membrane conductance

Defensin-like structures such as the defensins themselves and the lantibiotics show voltage-dependent formation of channels in artificial and biological membranes [17]. This voltage-dependence is shared by other bacteriocidins including colicins [13, 20,27]. At low voltages, the membrane conductance induced by these substances is rather small. However, at > 80 mV, there is a steep (exponential) dependence of the membrane conductance on the applied voltage. Therefore, we also investigated whether lactacin F-induced membrane conductance is voltage-dependent. Figure 4 shows the results of measurements following an increase ofthe voltage up to ± 100 mV. Lactacin F was added to the *cis*-side of the membrane in a concentration of 10 μg/ml. From ± 10 mV to ± 40 mV, the specific membrane conductance was approximately linear (G = 10 μs/cm²). At about 50 mV it started to increase, and at ± 100 mV it was about 50 times higher than at ± 10 mV, which suggested a considerable influence of membrane voltage on the conductive unit formed by lactacin F.

The application of even higher voltages resulted in a mechanical breakdown of the artificial membranes probably because of the formation of either too many or too large channels. The steep increase of conductance was approximately exponential (see Fig. 5), which may indicate that a defined number of gating charges is involved in the voltage dependence of the channels formed by lactacin F oligomers.

The current-voltage curve was only slightly asymmetric, even when the bacteriocidin was added to only one side of the membrane. This is in contrast to the membrane activity of a variety of similar small polypeptides, such as the lantibiotics, in which nisin in
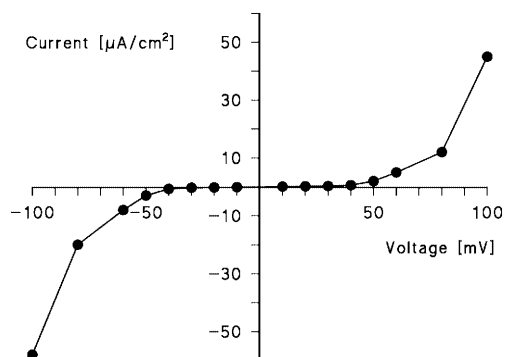
Fig. 4. Current-voltage relationship of lactacin F in DPhPC/*n*-decane lipid bilayer. Lactacin F was added at 10 μg/ml to the *cis*-side of black membranes. The voltage refers also to the *cis*-side. The aqueous phase contained 1 M KCl, pH 6; T = 20 °C. The means of five membranes are shown. Note the steep increase of the membrane current when the voltage exceeds 40 mV. Below 40 mV, the current-voltage curve was approximately linear with an average specific membrane conductance of about 10 μs/cm^2

Fig. 5. Dependence of the specific membrane conductance on the applied membrane potential on a semilogarithmic scale. The data were taken from Fig. 4. Note the almost exponential increase of the conductance as a function of voltage when the membrane voltage exceeds 50 mV

particular has a pronounced asymmetry and forms membrane channels only when the *trans*-side (the side opposite of the addition) has negative polarity [29]. Single-channel experiments at high voltage did not allow the clear resolution of single steps either because they were very rare at a sub-critical lactacin F concentration or because they were very frequent but with short lifetimes. Nevertheless, the channel had much higher amplitudes than at 10 mV. Lactacin F channels probably have a single-channel conductance of more than 10 ns at 1 M KCl and a voltage of 100 mV. This result indicates that the voltage shifts the equilibrium between non-conductive monomers and conductive oligomers in favor of the latter. This type of voltage-dependence is similar to that reported for nisin and other lantibiotics [9, 21, 29,31], except that lantibiotics often show asymmetric voltage-dependence in w hich the membrane conductance increases only when the *trans*-side is negative. Similar results have also been found for other bacteriocidins such as colicins [13,27]. Here we observed an almost symmetrical current-voltage relationship; lactacin F molecules probably do not need a special orientation of the membrane potential to enter the membranes. The great variations in the single-channel conductance under defined conditions suggests that the channel-forming units are not rigid structures like porins [10] or toxins, such as aerolysin of *Aeromonas sobria* or α-toxin of *Staphylococcus aureus* [12,23]. Instead, they undergo molecular changes that result in rapid current fluctuations of different conductance steps. Assuming that a complex of several lactacin F molecules is responsible for the formation of the conductive unit, the dissociation rate constant of the 1-ns channels is about 30 l/s. That a pore-forming oligomer is responsible for the action of lactacin F is supported by the steep conductance-concentration curve (see Fig. 1), which also suggests that several lactacin F molecules form the conductive unit, and that an association-dissociation equilibrium exists between non-conductive monomers
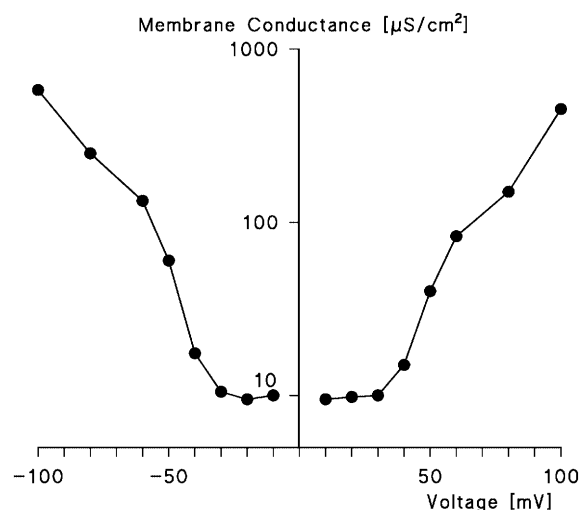
and conductive oligomers. The number of monomers does not seem to be fixed, otherwise the strong variation of the single-channel conductance could not be explained. The observation that lactacin F forms channels at low voltage is consistent with studies on different *Lactobacillus* strains and on *Enterococcus faecalis* cells, in which it has been demonstrated that uncouplers, which decrease the proton-motive force, do not stop the lactacin-F-mediated loss of potassium out of the cells [1]. The action of nisin and Pep 5 on de-energized cells, however, is very small or even absent at low membrane potential [21, 28–30].
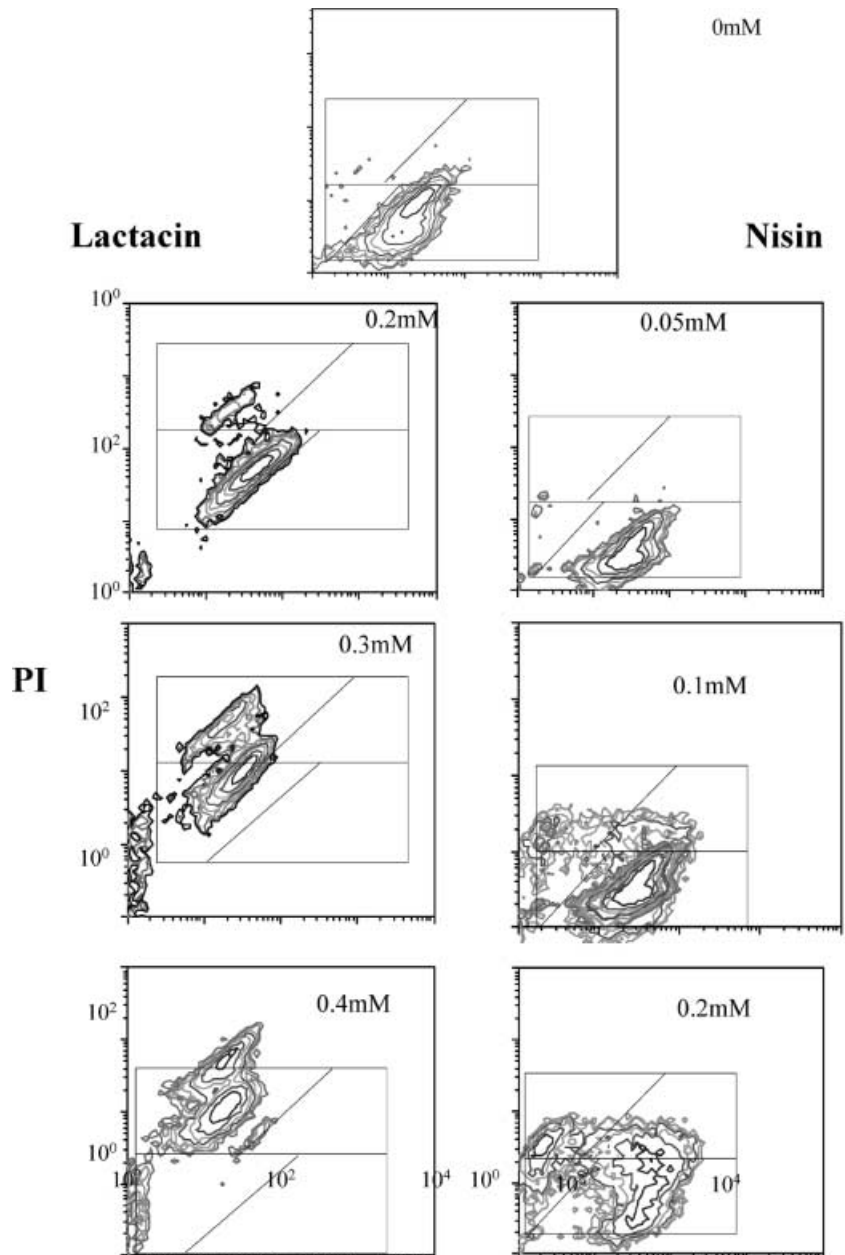
Zero-current membrane potential measurements

Zero-current membrane potential measurements were carried out to obtain further information on the molecular structure of lactacin F channels. After the incorporation of many channels into the PC membranes, the salt concentration on one side of the membranes was raised from 100 to 500 mM and the zero-current membrane potential was measured 5 min after the gradient was established. For all three salts employed in these experiments (KCl, LiCl and K-acetate), the most diluted side (100 mM) was always negative, which indicated preferential movement of the anions through the lactacin F channel at small transmembrane potential, i.e. the channel is anion-selective as was already suggested from the single-channel data. The zero-current membrane potential for the salts mentioned above was –12 mV (LiCl), –15 mV (KCl) and –20 mV (Kacetate) at the most diluted side of the membrane. Analysis of these data using the Goldman-Hodgkin-Katz equation [8] suggested that cations could permeate through the lactacin F channel because the ratios of the permeability

P_{cation} and P_{anion} were approximately among 0.7 (LiCl), 0.5 (KCl) and 0.3 (K-acetate). The results indicate that the ions move the same way inside the channel and in the aqueous phase, i.e. the lactacin F channel is indeed wide and water-filled as was already indicated by the single-channel conductance data.

Flow cytometry

Increasing concentrations of nisin (0.01, 0.05, 0.1, 0.2 and 1 mM) produced increasing damage to *L. helveticus* (Fig. 6). Injuries were also dependent on the time of exposure (data not shown). Similarly, lactacin F antibacterial action was also determined to be a function of length of contact and concentration.

When nisin-treated cells were stained with the Baclight kit and subsequently analyzed in the flow cytometer, a well-defined detection profile was observed. At low concentrations of nisin (or short exposure time), PI was incorporated into the cells. Later a "loss" of syto was detected, i.e. whereas control populations were syto+/PI–, after 30 min contact with 0.1 mM nisin most individuals were syto+/PI+. Longer exposures resulted in most of the bacteria being syto–/PI+. In the presence of lactacin, there was an initial loss of syto, whereas Pi was incorporated following longer exposures.

When cells were treated with both bacteriocins, the response was a combination of the changes detected when lactacin F and nisin were added separately, since some of the cells seemed to be affected by lactacin F and the rest seemed to be affected by nisin (Fig. 7).
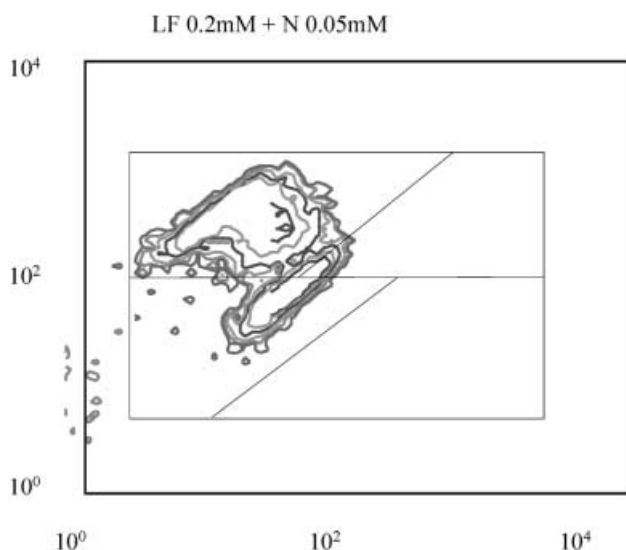
Fig. 6. Flow-cytometry profiles at increasing concentrations of nisin and lactacin

LF 0.2mM + N 0.05mM

Fig. 7. Flow-cytometry profiles when nisin (0.05 mM) and lactacin F (0.2 mM) were tested together

Finally, in order to confirm the influence of the proton-motive force in the bactericidal effect of lactacin F and nisin, the protonophore CCCP was added to the cultures. In control experiments with CCCP alone, the effect was similar to that obtained with lactacin F, i.e. a loss of syto was concomitant with CCCP addition. However, higher concentrations of CCCP alone did not lead to the entry of PI into the cells. This is a major handicap for these experiments since there was no way to determine whether the effects were due to lactacin F or to CCCP itself. However, in longer experiments in which CCCP and lactacin F were added together, PI became incorporated, which indicated that, even at low or almost no membrane potential, a certain number of lactacin channels were formed. When CCCP was added together with nisin, the results were clearly different since the average numbers of dead cells decreased from 23.8 to 8.5% after 10 min. Even in this case, however, the data should be considered with extreme caution due to the already mentioned effect of CCCP on cytometric profiles.

The extent of the injury induced by nisin and by lactacin F was time- and concentration-dependent, but the profiles were clearly different: treatment with nisin first led to the incorporation of PI, which indicates the formation of wide channels; further incubation would have produced populations in which only PI would have been detected. Taking into account that both dyes (syto 9 and PI) become linked to DNA (with different affinities, with PI having a higher affinity) it is feasible that longer exposures allows PI to displace syto from DNA. In contrast, after a short treatment with lactacin F (or at low concentrations) syto 9 did not enter the cell, most likely because the membrane potential fell rapidly when lactacin F was added. After longer exposures to lactacin F, PI also accumulated, and subsequently only PI-stained bacteria could be detected. Bacteriocins studies by flow cytometry should be interpreted carefully, taking into account these differences in response patterns. Note that in the presence of both nisin and lactacin F, the results are additive [25], with part of the population affected by nisin and the rest by lactacin F. Although this result is difficult to explain, it is clear that, at low concentrations, the two bacteriocins produced additive effects. Figure 7 shows an experiment in which cooperation between the two bacteriocins is demonstrated. In this case, cellular damage facilitates the entry of PI into the cells. This suggests some kind of cooperation. Two possible mechanisms could account for this: (1) both molecules cooperate to form either wider or more effective channels, and (2) the accumulation of injuries produced by each bacteriocin separately enhances the effects on the integrity of bacterial membranes. The first hypothesis does not seem probable because of the different chemical structures. This explanation would also be ruled out by the flow cytometry results, since different pores would lead to different flow cytometric patterns. The co-existence of both patterns of response to bacteriocins seems to support the second hypothesis, in which each bacteriocin acts independently, causing an increased number of membrane injuries that enhance the bacterial killing effect.

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