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Role of Lipid II and Membrane Thickness in the Mechanism of Action of the Lantibiotic Bovicin HC5[▽]

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Lantibiotics are antimicrobial peptides produced by Gram-positive bacteria, nisin being the most well-known member. Nisin inhibits peptidoglycan synthesis and forms pores at sensitive membranes upon interaction with lipid II, the essential bacterial cell wall precursor. Bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5, has the putative N-terminal lipid II binding motif, and we investigated the mode of action of bovicin HC5 using both living bacteria and model membranes, with special emphasis on the role of lipid II. Bovicin HC5 showed activity against *Staphylococcus cohnii* and *Staphylococcus warneri*, but bovicin HC5 hardly interfered with the membrane potential of *S. cohnii*. In model membranes, bovicin HC5 was not able to cause carboxyfluorescein release or proton influx from DOPC vesicles containing lipid II. Bovicin HC5 blocked lipid II-dependent pore formation activity of nisin, and a high-affinity interaction with lipid II was observed (apparent binding constant [K_a] = 3.1×10^6 M^{-1}), with a 1:1 stoichiometry. In DOPC vesicles containing lipid II, bovicin HC5 was able to assemble with lipid II into a prepore-like structure. Furthermore, we observed pore formation activity of bovicin HC5, which was stimulated by the presence of lipid II, in thin membranes. Moreover, bovicin HC5 induced the segregation of lipid II into domains in giant model membrane vesicles. In conclusion, bovicin HC5 has a primary mode of action similar to that of nisin, but some differences regarding the pore-forming capacity were demonstrated.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by species of *Bacteria* and certain strains of the *Archaea* domain (10). They differ in size, amino acid sequence, mechanisms of action, and range of antimicrobial specificities (7). Different peptides exhibit bactericidal or bacteriostatic activity, acting by inducing pore formation on sensitive cell membranes or inhibiting the peptidoglycan, nucleic acid, or protein synthesis (8).

Based on their primary structure, molecular mass, heat stability, and functional similarities, bacteriocins produced by Gram-positive bacteria were classified into four major groups (classes I to IV) (8). The bacteriocins belonging to class I are also named lantibiotics and, based on the differences on biosynthetic pathway among the peptides in this class, they were additionally divided into three subgroups: class I lantibiotics (peptides modified by LanB and LanC enzymes [nisin and epidermin]), class II lantibiotics (bacteriocins with a GG cleavage site in their leader peptide and that are modified by LanM enzymes [lacticin and its several analogous two-peptide lantibiotics]), and class III lantibiotics (peptides that have no or little antibacterial action and perform other functions, often related to morphogenesis [SapT, SapB, and their orthologues]) (29).

In general, lantibiotics are active in the nanomolar concentration range and kill bacteria by permeabilizing the plasma membrane, leading to the collapse of ion gradients across the cell membrane (27, 28). Efficient permeabilization is the result of pore formation, usually using a specific receptor (4, 12). Other peptides are also able to inhibit the bacterial cell wall synthesis without permeabilizing the membrane (13).

Nisin, the most well-known bacteriocin, is a class I lantibiotic produced by the lactic acid bacterium *Lactococcus lactis* subsp. *lactis*. It is a small (3.5-kDa) cationic and hydrophobic peptide, with five characteristic (beta-methyl) lanthionine rings. So far, nisin is the only bacteriocin which has been approved for use in over 50 countries as a food preservative (code E234), including the United States, the European Union, Australia, and New Zealand (9).

The antimicrobial activity of nisin is based on both low-affinity membrane and high-affinity target interactions. Nisin can disrupt microbial membranes by interacting with membrane phospholipids (4), and it is also able to displace or release enzymes, resulting in lysis of the cell wall (1, 14). Nisin binds with a high degree of affinity to the lipid II molecule, a hydrophobic carrier of peptidoglycan monomers, using this compound as a docking molecule for its pore-forming activity and leading to the inhibition of bacterial cell wall synthesis (3, 4, 5, 27). Nisin inhibits the outgrowth of bacterial spores, and recently, Gut and coworkers (11) showed that nisin utilizes lipid II as the target during inhibition of spore outgrowth and that the membrane disruption induced by nisin is important to inhibit the development of spores into vegetative cells.

Bovicin HC5 is a lantibiotic (2,449 Da) produced by *Streptococcus bovis* HC5, a lactic acid bacterium isolated from the bovine rumen. Bovicin HC5 contains posttranslational modified amino acids, is stable at high temperatures and low pH, and has a broad spectrum of activity, including against some food-borne microorganisms, such as *Listeria monocytogenes*

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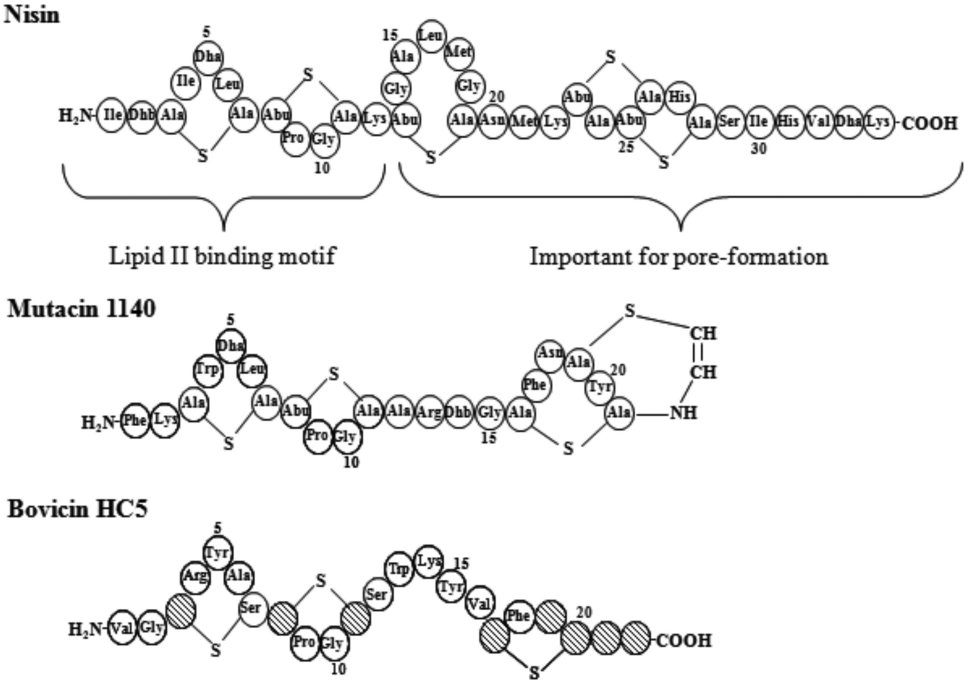

FIG. 1. Comparison of primary structures of nisin, mutacin 1140, and bovicin HC5. In nisin and mutacin structures, the posttranslationally modified amino acids residues, dehydroalanine (Dha) and deydrobutyrine (Dhb), as well as the lanthionine rings (Abu-S-Ala and Ala-S-Ala), are shown. In the bovicin HC5 structure, the residues indicated by striped circles do not correspond to any of the 20 amino acids commonly found in proteins, and they represent putative posttranslational modified amino acid residues; the suggested positions of lanthionine linkages are indicated by the lines.

(18, 19, 20). Increase in resistance against bovicin HC5 among sensitive bacteria has not been demonstrated until now.

Bovicin HC5 shares activity similarities with nisin, and the former peptide also carries the two lanthionine rings at its N terminus, which form the lipid II binding motif in the nisin molecule (Fig. 1). Based on these features, we hypothesized that bovicin HC5, like nisin, could target the essential bacterial cell wall precursor lipid II. In this paper, we characterized the lipid II-based activity of bovicin HC5 using live bacterial cells and artificial model membranes, and we compared the activities of bovicin HC5 and nisin.

MATERIALS AND METHODS

Chemicals and materials. Phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine ($C_{18:1}$) (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine ($C_{14:1}$) (DMoPC), and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine ($C_{12:0}$) (DLPC) were purchased from Avanti Polar Lipids, Inc. Phospholipids were dissolved in chloroform-methanol (1:1) to a stock concentration of 10 mM and stored at −20°C. After destruction of the phospholipids, the sample concentrations were determined by inorganic phosphate analysis, according to the procedure described by Rouser et al. (22).

Carboxyfluorescein (CF) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) (molecular weight [MW], 524.4) were purchased from Invitrogen. Lipid II with and without the pyrene label was synthesized and purified as described by Breukink et al. (3). All other chemicals used were of analytical or reagent grade.

Bacterial strains and culture conditions. The bovicin HC5-producing strain *Streptococcus bovis* HC5, previously isolated from bovine rumen (18), was cultivated under anaerobic conditions in basal medium containing (per liter) 0.292 g K_2HPO_4, 0.292 g KH_2PO_4, 0.48 g $(NH_4)_2SO_4$, 0.48 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.064 g $CaCl_2 \cdot 2H_2O$, 0.5 g cysteine hydrochloride, 4 g Na_2CO_3, 0.1 g Trypticase, and 0.5 g yeast extract at 39°C overnight. Glucose (4 g/liter) was added as a carbon source.

Staphylococcus cohnii and *Staphylococcus warneri* were grown in Mueller-Hinton medium (Sigma, St. Louis, MO) at 30°C, with aeration, for 5 h. Stock cultures were stored at −70°C, in solutions containing their own growth medium and 50% glycerol. Working cultures were maintained on agar plates and subcultured weekly.

Bacteriocins. Nisin A was isolated from commercial products (Chrisin [2.5% {wt/wt} nisin], 1,000 IU/mg; Christian Hansen, Denmark). Extracts of bovicin HC5 were prepared as described by Mantovani et al. (19). Purification of bovicin HC5 was performed by reverse-phase high-performance liquid chromatography (RP-HPLC) using a semipreparative column (Shimadzu C18; length, 150 mm; inner diameter, 4.6 mm; particle size, 5 μm). The column was equilibrated with buffer A (0.1% trifluoroacetic acid [TFA] in water), and the peptide was eluted using a linear gradient of 35 to 50% buffer B (80% acetonitrile, 0.1% TFA in water) at 22°C and at a flow rate of 1 ml min^{-1}. The absorbance was monitored at 214 and 280 nm, and the eluted fraction corresponding to pure bovicin HC5 was lyophilized. Analytical HPLC and electrospray mass spectrometry were used to confirm the correct mass and the purity of bovicin HC5.

Bacteriocin stock solutions (1 mM, in 0.05% acetic acid) were stored at −20°C until use. Protein concentration was determined using a bicinchoninic acid protein assay reagent (Pierce Chemical Corp., Bonn, Germany), with bovine serum albumin as a standard.

Susceptibility testing. The growth-inhibitory concentrations of bovicin HC5 and nisin were determined against *Staphylococcus cohnii*, a nisin-sensitive strain, and *Staphylococcus warneri*, which is a nisin-resistant strain. Overnight cultures of the strains were diluted 100-fold in Mueller-Hinton broth (optical density at 600 nm [OD_{600}] = 0.05). Different concentrations of purified bovicin HC5 and nisin in acetic acid (0.05%) were added, and the cultures were incubated at 30°C, with aeration; after 8 h of growth, the samples were collected and the OD_{600} was determined. The lowest antimicrobial agent concentration resulting in less than 1% outgrowth was determined and used in subsequent experiments. The results given are the means of three independent determinations.

Membrane depolarization assay using intact cells. The cytoplasmic membrane depolarization activity of bovicin HC5 and nisin was determined using the membrane potential-sensitive fluorophore 3,3′-diethylthiadicarbocyanine iodide [$DiSC_2(5)$] (15) and *Staphylococcus cohnii*. Bacterial cells in logarithmic phase (OD_{600} = 0.6 to 0.7) were centrifuged (1,742 × *g*, 15 min, 4°C), washed in 5 mM

HEPES buffer (pH 7.8) containing glucose (0.4%) and Mg^{2+} (2.5 mM), and ressuspended in the same buffer to an OD_{600} of 0.05 in a 1-cm quartz cuvette. The dye $DiSC_2(5)$ was added to a final concentration of 0.4 μM, followed by an incubation at room temperature for 2 min.

Subsequently, different concentrations of the bacteriocins were added and the fluorescence was followed over time with a SLM Aminco Spectrofluorometer (SPF-500C). An excitation wavelength of 622 nm and an emission wavelength of 650 nm were used.

Preparation of LUVs. Large unilamellar vesicles (LUVs) containing DOPC or DLPC-DMoPC (1:1) with and without lipid II were prepared for isothermal titration calorimetry, carboxyfluorescein leakage, and proton influx experiments by the extrusion technique (15). The dried lipid films were hydrated by the addition of 10 mM Tris and 150 mM NaCl (pH 7.5), of 10 mM Tris and 50 mM CF (pH 7.5), or of HPTS buffer (2 mM HPTS in 0.2 M NaH_2PO_4/Na_2HPO_4, pH 7.0), followed by vigorous stirring.

The LUVs were prepared by repeated extrusion through polycarbonate filters with a 0.2-μm pore size (Isopore membrane filters; Millipore, Ireland). Following the extrusion, vesicles were passed through a Sephadex G-50 spin column (equilibrated with 10 mM Tris and 150 mM NaCl [pH 7.5] or with 10 mM 2-morpholineethanesulfonic acid [MES] and 0.2 M Na_2SO_4 buffer [pH 5.5]) to remove the nonenclosed probe (CF or HPTS, respectively).

Vesicle concentrations were determined by phosphate determination, according to the method described by Rouser (22). In the case of HPTS vesicles, the lipids from the vesicles were first extracted according to the Bligh-Dyer procedure (2) in order to exclude the phosphate present in the buffer, and the inorganic phosphate determination was performed.

The final vesicle concentration used was 25 μM (final lipid-P_i), and the final concentration of lipid II varied according to the experiment performed (0.1 mol% for leakage experiments; 0.5 mol% for pyrene-labeled lipid II experiment; 2 mol% for isothermal titration calorimetry [ITC]).

Carboxyfluorescein leakage assay. Different concentrations of the bacteriocins were added to 10 mM Tris and 150 mM NaCl buffer (pH 7.5) containing CF-loaded vesicles (final concentration of 25 mM [lipid-P_i]). The CF leakage from the vesicles was monitored by measuring the fluorescence intensity at 516 nm (excitation wavelength at 492 nm), using a SLM Aminco spectrofluorometer (SPF-500C). The samples were continuously stirred in a quartz cuvette (10 by 4 mm), kept at 20°C using a continuous circulation water bath. Triton X-100 (final concentration of 0.2%) was added 2 min after the addition of the desired peptide, to disrupt the vesicles, and the resulting fluorescence was taken as the 100% leakage value.

The percentage of CF leakage was calculated according to the equation % of CF efflux = $(F_t - F_0)/(F_\infty - F_0) \times 100$, where F_t was the fluorescence at time t, F_0 was the fluorescence at time t_0 (baseline fluorescence for CF-loaded vesicles before the addition of peptides), and $F_\infty$was the maximum fluorescence (100%) after the addition of Triton X-100.

Proton permeability assay. The effects of the bacteriocins on the proton permeability was monitored by adding different concentrations of the peptides to 1 ml of 10 mM MES and 0.2 M Na_2SO_4 buffer (pH 5.5) containing HPTS-loaded vesicles (final concentration of 25 mM [lipid-P_i]). The fluorescence intensity was detected at 508 nm (excitation wavelength at 450 nm) on a SLM Aminco spectrofluorometer (SPF-500C). The samples were continuously stirred in a quartz cuvette (10 by 4 mm), kept at 20°C using a continuous circulation water bath. Triton X-100 was added after 2 min to disintegrate the vesicles, and the resulting fluorescence was taken as 100% of proton influx.

Isothermal titration calorimetry (ITC) measurements. Titration experiments were performed on an Auto-iTC_{200} isothermal titration calorimeter (MicroCal Inc.). DOPC LUVs with or without 2 mol% lipid II were prepared as described, in 10 mM Tris and 150 mM NaCl (pH 7.5). The vesicles were injected into the sample cell (volume, 0.2 ml) containing 20 μM bovicin HC5 in the same buffer used for the vesicles. All solutions were degassed before starting the experiment.

The ITC runs consisted of 16 injections, 5 μl each, of a vesicle stock solution at 25°C (20 mM final phospholipid concentration), and the experiment proceeded over 33 min. The results were analyzed using the Origin software (version 7.0) provided by MicroCal Inc.

Assessing assembly using pyrene-labeled lipid II. LUVs were prepared in 10 mM Tris and 150 mM NaCl (pH 7.5), and the fluorescence spectrum was recorded between 360 and 550 nm (excitation wavelength at 350 nm) in the absence and presence of the bacteriocins after an incubation time of 5 min, on a SLM Aminco spectrofluorometer (SPF-500C); the samples were continuously stirred in a quartz cuvette (10 by 4 mm), kept at 20°C using a water bath with continuous circulation.

To prevent the influence of quenching effects of the peptides on the monomer fluorescence of pyrene lipid II on the excited state dimer (excimer)/monomer ratios, these were calculated using the monomer fluorescence on the sample in the absence of peptide, as described before (12).

Preparation of GUVs using electroformation. Giant unilamellar vesicles (GUVs) were prepared using a homemade electroformation setup with ITO coverslips (21). On one slip, 5 μl from a chloroform-methanol (1:1, vol/vol) solution containing 2.5 mM DOPC and 0.2 mol% NBD-labeled lipid II was deposited and dried in a vacuum desiccator (1 h), in order to remove the remaining solvents. With this slip, the electroformation chamber was assembled and filled with a solution of 100 mM sucrose.

Via two clamps, the GUV cell was connected to the power supply, and an alternating current was applied (3 V, 10 Hz). GUV formation was carried out for 3 h, and the chambers were analyzed directly using confocal fluorescence microscopy.

Peptides were added by pipetting the desired volume from a 1 mM solution directly into the GUV chamber.

Confocal fluorescence microscopy. The NBD label was detected using an argon laser (488 nm; Spectra-Physics), and it appeared green upon excitation. Difference interference contrast (DIC) (Nomarski optics) was used for detection of GUVs, as a control for their shape.

Nikon EZ-C1 software, version 2.20 Gold, was used for analysis of the images.

RESULTS

Antimicrobial activity of bovicin HC5 and nisin. Bovicin HC5 and nisin have distinct amino acid sequences, but the two peptides share structural similarities in their N-terminal backbone, specially the location of the lanthionine rings and the amides involved in the intermolecular hydrogen bonds that are important for the formation of the pyrophosphate cage that mediates lantibiotic binding to lipid II (Fig. 1). Nisin and bovicin HC5 inhibit many Gram-positive bacteria, but their activities vary among bacterial species. When *S. cohnii*, a nisin-sensitive bacterium, was exposed to increasing concentrations of nisin and bovicin HC5, growth inhibition was achieved at 0.05 μM and 0.2 μM, respectively.

However, 10 μM nisin and 2 μM bovicin were required to inhibit the growth of *S. warneri*, a nisin-resistant strain. Nisin-treated cultures were able to resume growth after 24 h of incubation and reached maximum OD_{600}s of 3.3 and 4.4 for *S. cohnii* and *S. warneri*, respectively. In contrast, regrowth was not observed for bovicin HC5-treated cells even after 48 h of incubation at the concentrations reported above. These results suggested that the interaction between bovicin HC5 and the target cells was different from that between the target cells and nisin.

Membrane depolarization. To verify whether bovicin HC5 caused disruption of the membrane potential of living bacterial cells, membrane depolarization assays were performed with exponentially growing *S. cohnii* cultures, and the fluorescence of $DiSC_2(5)$ was used as an indicator for the presence of a membrane potential.

Despite the high sensitivity to both bacteriocins, neither bovicin HC5 nor nisin caused significant membrane depolarization in *S. cohnii* cells (Fig. 2, lines A and B). Upon addition of 4 μM bovicin HC5 (20 times the lowest inhibitory concentration), only a minor effect in the membrane potential of *S. cohnii* could be observed (Fig. 2, line C). Nisin was more efficient in dissipating the membrane potential of *S. cohnii*, since at 0.2 μM (final concentration) this peptide caused major release of the dye from the *S. cohnii* membrane, showing that the membrane potential was dissipated upon the addition of nisin (Fig. 2, line D).

These results suggest that bovicin HC5, even at micromolar

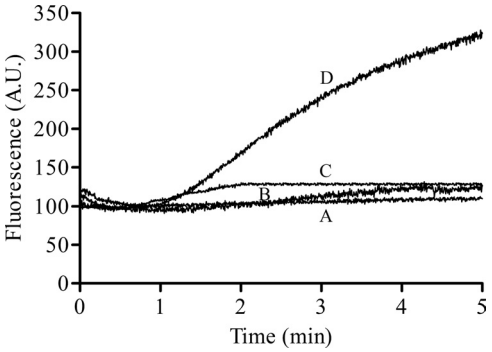

FIG. 2. Effect of bovicin HC5 and nisin on the membrane potential of glucose-energized cells of *Staphylococcus cohnii*, determined using $DiSC_2(5)$, a membrane potential-sensitive fluorophore. Lines: A, 0.2 μM bovicin HC5; B, 0.05 μM nisin; C, 4 μM bovicin HC5; D, 0.2 μM nisin. The bacterial cells were diluted to an OD_{600} of 0.05, and $DiSC_2(5)$ was added to a final concentration of 0.4 μM. The bacteriocin addition was performed when a baseline was reached. Dye release was monitored at excitation and emission wavelengths of 622 and 650 nm, respectively. A.U., arbitrary units.

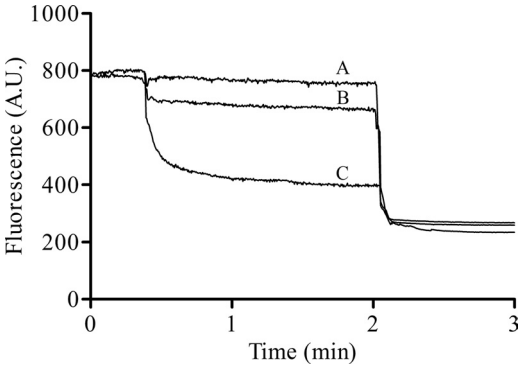

FIG. 4. Effect of bovicin HC5 and nisin on the proton permeability of DOPC lipid II containing vesicles. Time courses of HPTS fluorescence are shown after addition of the bacteriocins to model membranes: line A, 0.1 μM bovicin HC5; line B, 1 μM bovicin HC5; line C, 0.1 μM nisin. Measurements were performed in 10 mM MES, 0.2 M Na_2SO_4 buffer (pH 5.5), and DOPC lipid II vesicles (25 μM final lipid-P_i). A.U., arbitrary units.

concentration, does not cause a significant membrane disruption and uses an alternative mechanism of action to exert its bactericidal activity against the sensitive bacterial cells.

Specific interaction with lipid II. To determine if bovicin HC5, like nisin, could exhibit lipid II-binding ability, model membranes containing lipid II and different lipid compositions were used in several different assays. The CF leakage assay was initially performed to investigate the pore-forming capacity of bovicin HC5 in neutral conditions (pH 7.5), using DOPC vesicles.

In DOPC/DOPG vesicles with a negative surface charge, and in liposomes made of pure DOPC, bovicin HC5 or nisin did not cause CF efflux, even at a concentration of 1 μM (data not shown). In the presence of DOPC vesicles containing 0.1 mol% lipid II, the addition of 0.1 μM or 1 μM bovicin HC5 (Fig. 3, lines A and B) did not result in significant efflux activity (about 10% when 1 μM bovicin HC5 was used). This strongly contrasted with the effect of 0.1 μM nisin, which caused an immediate leakage at CF-loaded DOPC/lipid II vesicles, due to the lipid II-dependent pore formation capacity of nisin; in this case, a leakage of 80% was observed (Fig. 3, line C).

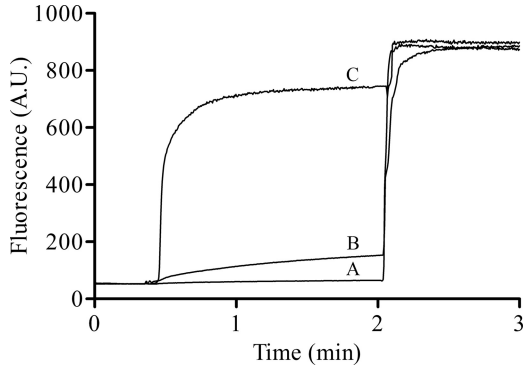

FIG. 3. Activity of 0.1 μM (line A) and 1 μM (line B) bovicin HC5 on DOPC lipid II vesicles. The fluorescence of samples containing CF-loaded DOPC vesicles with 0.1 mol% lipid II was recorded for 3 min. Nisin or bovicin HC5 was added after 20 s, and Triton X-100 solution was added to yield the 100% leakage value. The activity of 0.1 μM nisin (line C) is shown as a positive control. A.U., arbitrary units.

Some antibiotics are known to form small pores on sensitive membranes, and a similar situation could be the case for bovicin HC5. To check the possibility that bovicin HC5-induced pores could be smaller than the pores formed by nisin and too narrow to allow CF leakage (1 nm of molecular radius), we performed a proton influx assay, using HPTS. This is a fluorescent compound that can be used as a pH-dependent probe, and the influx of H^+ can be measured by the amount of quenching obtained (26).

The ability of bovicin HC5 and nisin to dissipate the applied proton gradient was measured as a change in HPTS emission. No decrease of fluorescence intensity was observed when the bacteriocins were added to DOPC-containing vesicles (25 μM lipid-P_i) in the absence of lipid II (data not shown). The addition of 0.1 μM bovicin HC5 to HPTS-loaded DOPC/lipid II vesicles did not result in proton influx (Fig. 4, line A), but the influx of H^+ was observed when 1 μM bovicin HC5 was used (Fig. 4, line B). The addition of nisin (0.1 μM final concentration) resulted in an immediate dissipation of the proton gradient over the DOPC lipid II-containing vesicles (Fig. 4, line C), and this effect was approximately 4-fold higher than the effect obtained with 1 μM bovicin HC5.

The results obtained with CF and HPTS assays show that bovicin HC5, in contrast to nisin, did not induce significant leakage in DOPC/lipid II vesicles, even in the micromolar range, which indicates that bovicin HC5 does not perturb the membrane barrier in DOPC vesicles in a lipid II-dependent way.

To check if bovicin HC5 was able to interact with lipid II even in the absence of leakage, we performed competition assays by sequential addition of nisin after bovicin HC5. A similar approach had previously been used with vancomycin in the study that determined the capacity of nisin (4) and mutacin (24) to bind to lipid II.

Similar to the results presented in Fig. 3, a CF leakage of 80% is observed when 0.1 μM nisin is added to DOPC vesicles

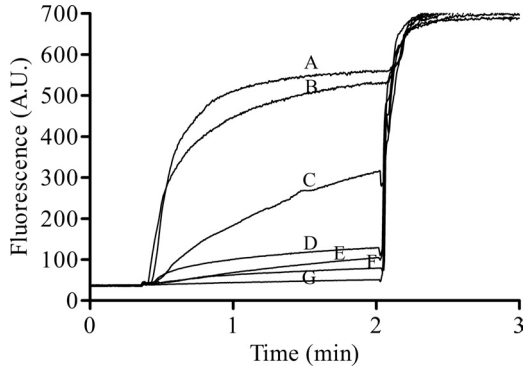

FIG. 5. Interference of lipid II-dependent pore formation activity of nisin by bovicin HC5. Different concentrations of bovicin HC5 (0.01 to 0.1 μM) were added 20 s before nisin addition (0.1 μM final concentration) to DOPC vesicles (25 μM lipid-P_i) containing 0.1 mol% lipid II. CF leakage was monitored for 2 min, and after that, Triton X-100 was added to determine the maximum CF leakage. Lines: A, 0.1 μM nisin; B, 0.01 μM bovicin HC5; C, 0.02 μM bovicin HC5; D, 0.03 μM bovicin HC5; E, 0.04 μM bovicin HC5; F, 0.05 μM bovicin HC5; G, 0.1 μM bovicin HC5. A.U., arbitrary units.

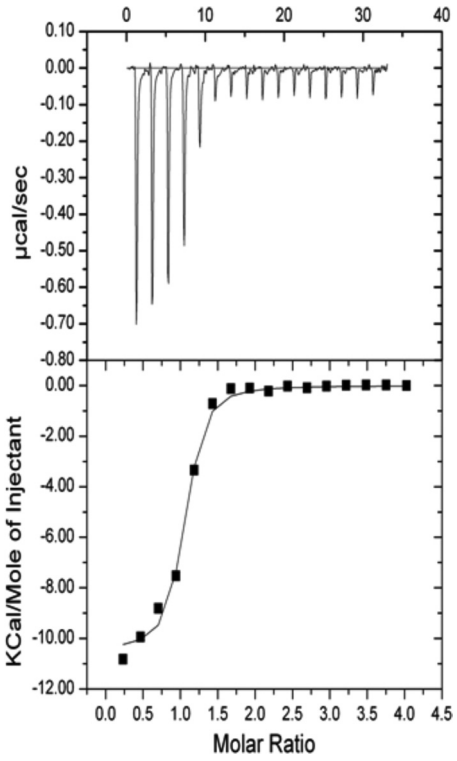

FIG. 6. Calorimetric titrations of bovicin HC5 with DOPC vesicles containing 2 mol% lipid II. Vesicles were dissolved in 10 mM Tris and 150 mM NaCl (pH 7.5). The graph on the top shows the heat peaks after injections of 5 μl vesicles (20 mM final phospholipid concentration) into the sample cell containing 20 μM bovicin HC5 in the same buffer. The bottom graph displays the integrated heat per injection, normalized to the injected amount of moles of lipid II and displayed against the molar ratio of lipid II versus bovicin HC5.

containing lipid II (Fig. 5, line A). In the presence of 0.02 μM bovicin HC5, CF leakage was significantly reduced by 50% (Fig. 5, line C), and increasing concentrations of bovicin HC5 resulted in higher reductions until complete inhibition of nisin-induced CF leakage was obtained at 0.1 μM bovicin HC5 (Fig. 5, line G).

These results clearly show that bovicin HC5 was able to bind to lipid II. This binding inhibited the pore-forming activity of nisin, most likely because lipid II binding sites were already occupied by bovicin HC5, which indicates that both bacteriocins compete for the same receptor and bovicin HC5 binds with high affinity to the lipid II, which becomes unavailable to nisin binding.

In order to gain more insight into the interaction between bovicin HC5 and lipid II, ITC measurements were performed with DOPC vesicles with and without lipid II, which were titrated into a solution of 20 μM bovicin HC5. In the absence of lipid II, bovicin HC5 displayed little interaction with the DOPC vesicles (data not shown). However, in the presence of 2 mol% lipid II-containing LUVs, significant interaction between bovicin HC5 and lipid II was evidenced by the heat effects, and a clear saturation of the interaction could be observed above a molar ratio of 1.5 (Fig. 6). The apparent binding constant (K_a) between bovicin HC5 and lipid II was estimated to be 3.1×10^6 M^{-1}, and the stoichiometry of this binding was determined to be 1:1, when all the amount of lipid II present at the vesicles was considered.

In order to investigate if bovicin HC5 could assemble into a prepore-like structure (together with lipid II), as was shown for a nisin hinge mutant that lacked the ability to form pores (12), we used pyrene-labeled lipid II. This same approach was previously used to characterize the nisin-lipid II pore complex (3, 12).

Pyrene-labeled lipid II can be used as a sensor of pore assembly, because pyrene shows a distance-dependent signal: pyrene monomers exhibit characteristic fluorescence emission maxima at three wavelengths (378, 398, and 417 nm). In addition, pyrene can display a fluorescence peak around 495 nm, which occurs when two pyrene rings become stacked at a distance of 0.35 nm from each other and form an excited state dimer, or excimer (25).

The addition of bovicin HC5 to pyrene-labeled lipid II-containing DOPC vesicles caused a large decrease of monomer signal intensity, likely due to the interaction of bovicin HC5 with lipid II. However, only a small increase, not proportional to the reduction at monomer signals, was determined between 480 and 510 nm, where the excimer fluorescence was expected to be observed (Fig. 7A).

The reduction of monomer fluorescence caused by the addition of bovicin HC5 had an extent similar to that of the reduction caused by nisin, but the induced excimer formation was more evident with nisin. A more quantitative picture is presented in Fig. 7B, where the ratio between monomer and excimer signals (E/M) is depicted as a function of the peptide concentration. The maximal ratios that were obtained were 0.018 and 0.050 for bovicin HC5 and nisin, respectively. These plateau values were reached at concentrations of 0.1 μM bovicin HC5 and 0.2 μM nisin (Fig. 7B).

These latter results indicated that bovicin HC5 does assemble into some kind of complex with lipid II, but there might be some differences between bovicin HC5 and nisin with respect to the pore-forming ability. Aiming to investigate if this assem-

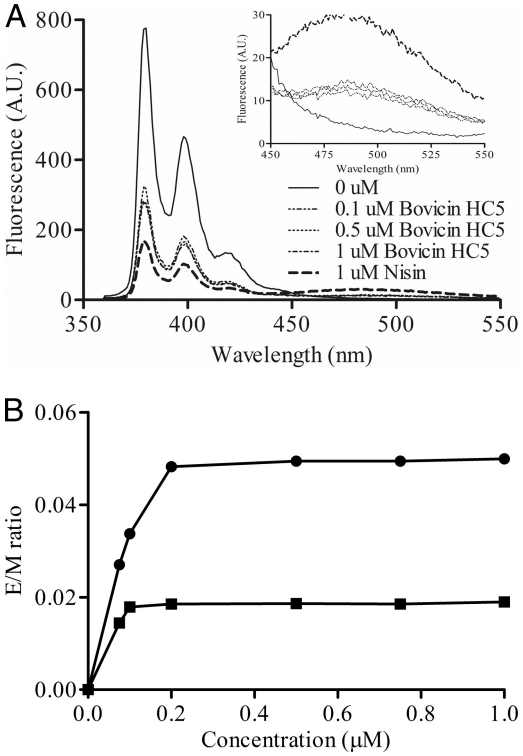

FIG. 7. (A) Fluorescence spectra of pyrene-labeled lipid II (0.5 mol%) in DOPC vesicles (25 μM lipid-P_i). Pyrene-labeled lipid II fluorescence was recorded in the absence of bovicin HC5 and after incubation for 5 min with different concentrations of bovicin HC5 (0.1, 0.5, and 1 μM). The fluorescence spectra in the absence of bacteriocins (0 μM) and after the addition of 1 μM nisin are shown as negative and positive controls, respectively. The inset shows an enlarged view of the excimer emission part of the spectra. Spectral recordings were performed between 360 and 550 nm, and at an excitation wavelength of 350 nm. (B) Ratio between monomer and excimer signals (E/M ratio) of pyrene-labeled lipid II obtained for DOPC LUVs after the addition of nisin (circles) and bovicin HC5 (squares). The E/M ratio is shown as a function of bacteriocin concentration.

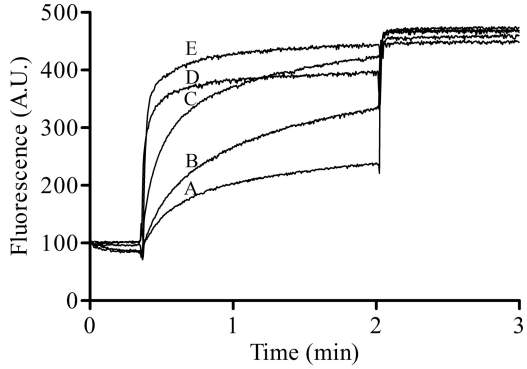

FIG. 8. Activity of bovicin HC5 (line A, 0.1 μM; line C, 1 μM) and nisin (line E, 0.1 μM) on DLPC/DMoPC lipid II vesicles. Activity of 1 μM bovicin HC5 (line B) and 0.1 μM nisin (line D) on DLPC/DMoPC vesicles without lipid II are also shown. Fluorescence of samples containing CF-loaded DLPC/DMoPC vesicles with 0.1 mol% lipid II was recorded for 3 min. Nisin or bovicin HC5 was added after 20 s, and the 100% leakage level was determined by the addition of Triton X-100. A.U., arbitrary units.

bly involved a prepore complex or if the membranes were too thick to allow actual pore formation, we decided to check the possible lipid II-mediated pore formation of bovicin HC5 using another membrane system, composed of phospholipids with shorter acyl chain length, DLPC/DMoPC (1:1, $C_{12:0}$-$C_{14:1}$). Such thin membranes were stable, and no spontaneous CF leakage was detected.

A different picture emerged upon testing the effects of the lantibiotics on the shorter acyl chain length membrane system, compared to the results above, obtained with DOPC vesicles. In the presence of lipid II, considerable CF leakage was detected when bovicin HC5 was added to the DLPC/DMoPC vesicles (0.1 μM and 1 μM; Fig. 8, lines A and C). However, even in the absence of lipid II, leakage of CF was observed when 1 μM bovicin HC5 was added (Fig. 8, line B). The addition of 0.1 μM nisin caused more than 80% or 95% of CF leakage from DLPC/DMoPC vesicles, without and with 0.1 mol% lipid II, respectively (Fig. 8, lines D and E).

The leakage of DLPC/DMoPC vesicles caused by bovicin HC5 was stimulated by the presence of lipid II, although the rate and the efficiency of CF release were lower than the leakage caused by nisin in the presence or absence of lipid II. Since lipid II-mediated pore formation by bovicin HC5 is dependent on the membrane thickness, we decided to check whether the segregation of lipid II into domains could be part of the bovicin HC5 mode of action, as already demonstrated for mutacin 1140 and mutants of nisin (13, 27).

We visualized the bovicin HC5 capacity of assembly with lipid II into a complex in a macro scale using GUVs composed by DOPC and 0.2 mol% NBD-labeled lipid II. The GUVs were prepared in homemade chambers and analyzed directly on a confocal microscope. Bovicin HC5 or nisin (a few μl from a 1 mM stock solution) was added to one edge of the sample chamber, and each diffused into the field of focus.

Before the addition of peptides to the chamber, the GUVs had a uniform fluorescent membrane, as a consequence of the homogeneous distribution of NBD-labeled lipid II over the membrane (Fig. 9A and 10A).

As already demonstrated by Hasper et al. (13), after 2 min of exposure to 20 μM nisin, the green fluorescence used started to segregate into patches (Fig. 9B), and after 7 min, the typical lipid II patches were obtained (Fig. 9C). Moreover, after 10 min of exposure to nisin, the vesicles in close proximity started to adhere to each other, the adhesion sites became more fluorescent, and the rest of the fluorescence remained spread into patches over the membrane (Fig. 9D). Those results confirmed the sequestration of lipid II into large domains induced by nisin, as a result of the high-affinity interaction between this lantibiotic and its receptor in membranes.

The addition of bovicin HC5 to the GUVs also resulted in a heterogeneous spreading of the fluorescence, but a different picture was obtained. After 5 min of exposure to 20 μM bovicin HC5, the vesicles changed their common shape (Fig. 10B). However, the lipid II sequestration into separate domains was observed only when the vesicles were exposed for long periods of time to the same concentration of bovicin HC5 (30 min; Fig. 10C) or if larger amounts of the peptide (40 μM) were used (Fig. 10D and E).

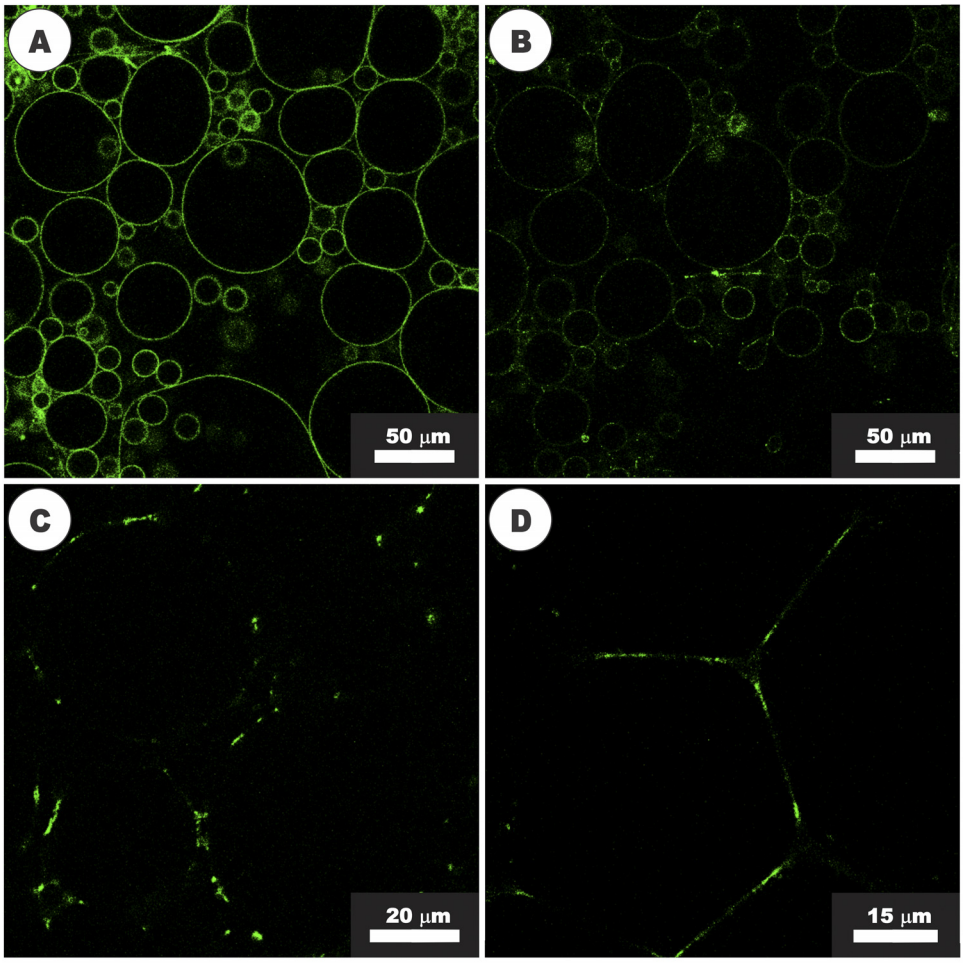

FIG. 9. Activity of nisin (20 μM) visualized with confocal fluorescence microscopy. (A) GUVs composed of DOPC and NBD-labeled lipid II before the addition of nisin. (B) GUVs after 2 min of exposure to nisin, showing the beginning of lipid II segregation process. (C) After 7 min of nisin activity, the clustering of lipid II into large domains was observed. (D) Adhesion of GUVs in close proximity, after 10 min of exposure to nisin.

DISCUSSION

In the present study, we show that bovicin HC5 has a primary mode of action similar to that of nisin, which involves specific interaction with the cell wall precursor lipid II. However, some differences regarding the pore-forming capacity of both bacteriocins were demonstrated.

Bovicin HC5 was able to inhibit nisin-sensitive and nisin-resistant bacteria, but the patterns of activity were quite different from that with nisin-treated cells. Bovicin HC5 shows a more persistent antibacterial activity, inhibiting the growth of the indicator strains even after 24 h of incubation. Bovicin HC5 was also 5-fold more effective than nisin in inhibiting the growth of the nisin-resistant bacterium *S. warneri*. However, despite the sensitivity, the membrane potential in intact *S. cohnii* cells was only slightly affected by high concentrations of bovicin HC5, which contrasted with the effects caused by nisin (Fig. 2).

Bovicin HC5 did not produce significant CF leakage or proton influx from liposomes composed of DOPC and lipid II (Fig. 3 and 4), while nisin caused an efficient and rapid CF release and H^+ influx from the vesicles, even at low concentrations (0.1 μM). Nevertheless, independent of the presence of detectable leakage, bovicin HC5 was able to bind to lipid II, and this binding was confirmed when competition assays and ITC experiments were performed (Fig. 5 and 6). The ITC results show that bovicin HC5 bound with an apparent high affinity to lipid II ($K_a = 3.4 \times 10^6\ M^{-1}$), with a stoichiometry of 1:1 (Fig. 6).

Taken together, these results indicate that bovicin HC5 efficiently binds to lipid II and the stability of this interaction is strong enough to prevent an interaction between nisin and its target. A possible explanation for this high affinity to lipid II is that bovicin HC5 has an arginine in position 4 (instead of isoleucine in nisin), which provides an additional positive charge to the putative lipid II motif and might enhance binding to the pyrophosphate moiety of lipid II, considered the primary binding site for nisin (16).

Using pyrene-labeled lipid II we show that bovicin HC5 is able to bring some lipid II molecules into close proximity, assembling into a prepore-like structure (Fig. 7). Mutacin 1140 and mutants of nisin that lack the hinge region are also not able to form pores on DOPC vesicles containing lipid II,

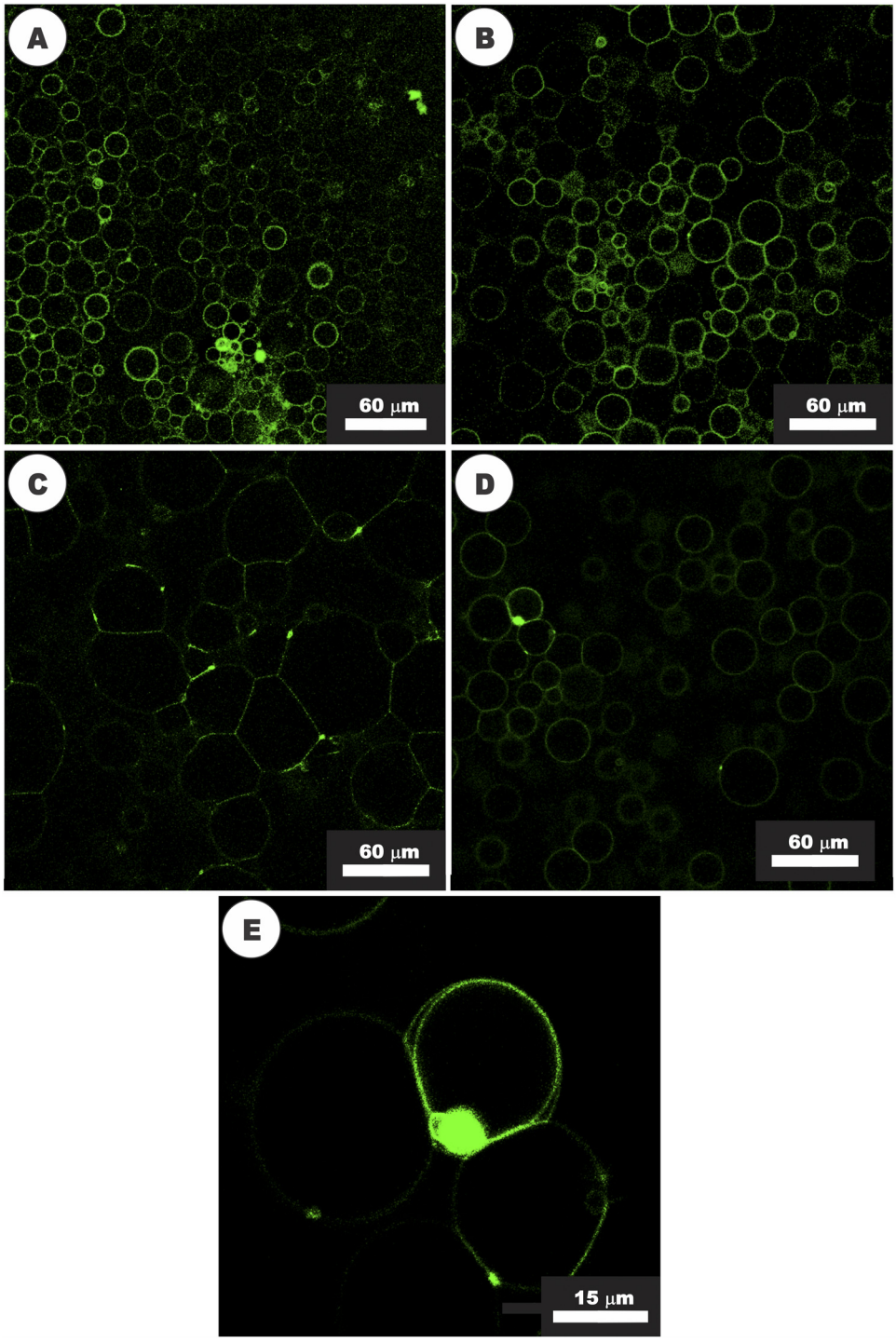

FIG. 10. Activity of bovicin HC5 visualized with confocal fluorescence microscopy. (A) GUVs composed of DOPC and NBD-labeled lipid II before the addition of bovicin HC5. (B) Changing of the common shape of GUVs, after 5 min of exposure to bovicin HC5 (20 μM). (C and D) After 30 min of bovicin HC5 activity (C) or at higher concentrations of this peptide (40 μM) (D), the segregation of lipid II into domains was observed. (E) An enlarged view of the clustering of lipid II into domains caused by bovicin HC5 (40 μM).

but they cause some pyrene excimer fluorescence by assembling with lipid II into a prepore-like structure (24, 27).

The structure of bovicin HC5/lipid II complex has not yet been resolved, but it seems to be different from that of the nisin/lipid II complex. In the presence of bovicin HC5, the orientation of the pyrenes within the pore is probably different, and they stack to a much lesser extent than in the pore formed by nisin (the distance between two lipid II molecules within the pore complex formed by nisin was estimated to be 18 Å [3]), leading to a lower excimer signal.

The thickness of the lipid bilayer in liposomes composed of monounsaturated phosphorylcholines depends on the number of acyl chain carbons and can be estimated by the equation $dL = (15.9 \pm 1.4) + (1.5 \pm 0.2)n$ Å, where dL is the bilayer thickness and n is the number of carbon atoms of the acyl chain (17). Thus, we can assume that the thickness of the membranes composed of DOPC was approximately 43 Å. Nisin has an overall length of 50 Å, while the predicted bovicin HC5 length is 33 Å. This observation suggests that bovicin HC5 is too short to permeabilize thick lipid bilayers composed of phospholipids with $C_{18:1}$ or longer acyl chains.

This hypothesis was confirmed when the bovicin HC5 activity on vesicles composed of DLPC/DMoPC was determined (Fig. 8). Under such conditions, bovicin HC5 was able to form pores, causing the efflux of CF from the liposomes, which was more efficient when lipid II was present in the bilayer. According to the equation described by Kučerka et al. (17), the thickness of the membranes composed of DLPC/DMoPC can be estimated to be 38 Å, shorter enough to be spanned by bovicin HC5.

This membrane thickness dependency has also been demonstrated for the pore formation capacity of gallidermin. Gallidermin, a peptide that has 22 amino acid residues and an inflexible C terminus (forming a closed structure due to a lanthionine ring), also interacts with lipid II, and its pore formation capacity was observed only in model membranes composed of short-chain phospholipids (7). More recently, Christ et al. (6) demonstrated that besides membrane thickness, the composition of the sensitive membrane, such as the presence of branched membrane lipids, can modulate the membrane properties and facilitate the access to lipid II by gallidermin.

Nontargeted amphiphilic peptides, such as magainin and dermaseptin, do not form a defined pore structure on sensitive cell membranes, but instead they can be present in multiple aggregational states (23). This can also be the case when high concentrations of nisin and bovicin HC5 are used on thin membranes, which suggests that both bacteriocins are able to disturb the membrane integrity independent of lipid II.

Other studies indicated that bovicin HC5 might impair membrane functions. It was previously visualized through atomic force microscopy that the treatment of sensitive cells with bovicin HC5 induces severe cell shape deformations (H. C. Mantovani, unpublished data), and these characteristics are typical for compounds that interfere with cell wall biosynthesis. In this study, we demonstrated that bovicin HC5 also induces the segregation of lipid II into domains in giant model membranes (Fig. 10), a mechanism that strengthened the idea that bovicin HC5 interferes with cell wall synthesis.

When used together, bovicin HC5 and nisin show additive effects and they inhibit sensitive bacteria in a more efficient way than when they are used separately (Mantovani, unpublished). Moreover, bacteria that can readily become resistant to nisin did not become significantly resistant to bovicin, even after they were repeatedly transferred with sublethal doses of the peptide (20), suggesting that these two lantibiotics, although sharing the same target, still have different modes of action or different mechanisms to reach their targets.

In this study, we determined the mode of action of bovicin HC5 based on the interaction with its specific target, lipid II. The pore-forming activity of bovicin HC5 is clearly dependent on membrane thickness and stimulated by the presence of lipid II. However, bovicin HC5 maintains its antibacterial activity independent of the membrane thickness, by binding to lipid II and recruiting some lipid II molecules as a prepore-like structure. Besides those activities, bovicin HC5 acts by sequestering lipid II, inhibiting bacterial cell wall biosynthesis. These varied mechanisms can be combined on sensitive cells and might explain the differences observed in sensitivity to the lantibiotics depending on the bacterial strain tested.

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REFERENCES

1. **Bierbaum, G., and H. G. Sahl.** 1985. Induction of autolysis of staphylococci by the basic peptide antibiotic pep5 and nisin and their influence on the activity of autolytic enzymes. Arch. Microbiol. **141:**249–254.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
3. **Breukink, E., et al.** 2003. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J. Biol. Chem. **278:**19898–19903.
4. **Breukink, E., et al.** 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science **286:**2361–2364.
5. **Brötz, H., et al.** 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol. **30:**317–327.
6. **Christ, K., et al.** 2008. Membrane lipids determine the antibiotic activity of the lantibiotic gallidermin. J. Membr. Biol. **226:**9–16.
7. **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. **71:**1–20.
8. **Cotter, P. D., C. Hill, and R. P. Ross.** 2005. Bacterial lantibiotics: strategies to improve therapeutic potential. Curr. Protein Pept. Sci. **6:**61–75.
9. **Delves-Broughton, J.** 2005. Nisin as a food preservative. Food Aust. **57:**525–527.
10. **Drosinos, E. H., M. Mataragas, and J. Metaxopoulos.** 2006. Modeling of growth and bacteriocin production by Leuconostoc mesenteroides E131. Meat Sci. **74:**690–696.
11. **Gut, I. M., S. R. Blanke, and W. A. van der Donk.** 2011. Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. ACS Chem. Biol. **6:**744–752.
12. **Hasper, H. E., B. de Kruijff, and E. Breukink.** 2004. Assembly and stability of nisin-lipid II pores. Biochemistry **43:**11567–11575.
13. **Hasper, H. E., et al.** 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science **313:**1636–1637.
14. **Héchard, Y., and H. G. Sahl.** 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. Biochimie **84:**545–557.
15. **Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis.** 1985. Production of large unilamellar vesicles by a rapid extrusion procedure—characterization of size distribution, trapped volume and ability to maintain a membrane-potential. Biochim. Biophys. Acta **812:**55–65.
16. **Hsu, S. T., et al.** 2004. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat. Struct. Mol. Biol. **11:**963–967.
17. **Kučerka, N., D. Uhríková, J. Teixeira, and P. Balgavý.** 2004. Bilayer thickness in unilamellar phosphatidylcholine vesicles: small-angle neutron scattering using contrast variation. Physica B **350:**e639–e642.
18. **Mantovani, H. C., D. K. Kam, J. K. Ha, and J. B. Russell.** 2001. The antibacterial activity and sensitivity of *Streptococcus bovis* strains isolated from the rumen of cattle. FEMS Microbiol. Ecol. **37:**223–229.
19. **Mantovani, H. C., H. Hu, R. W. Worobo, and J. B. Russell.** 2002. Bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. Microbiology **148:**3347–3352.
20. **Mantovani, H. C., and J. B. Russell.** 2003. Inhibition of *Listeria monocytogenes* by bovicin HC5, a bacteriocin produced by *Streptococcus bovis* HC5. Int. J. Food Microbiol. **89:**77–83.
21. **Ramadurai, S., et al.** 2010. Influence of hydrophobic mismatch and amino acid composition on the lateral diffusion of transmembrane peptides. Biophys. J. **99:**1447–1454.

22. **Rouser, G., S. Fkeischer, and A. Yamamoto.** 1970. Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids **5:**494–496.
23. **Shai, Y.** 1999. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell nonselective membrane-lytic peptides. Biochim. Biophys. Acta **1462:**55–70.
24. **Smith, L., et al.** 2008. Elucidation of the antimicrobial mechanism of mutacin 1140. Biochemistry **47:**3308–3314.
25. **Somerharju, P.** 2002. Pyrene-labeled lipids as tools in membrane biophysics and cell biology. Chem. Phys. Lipids **116:**57–74.
26. **van Kan, E. J., R. A. Demel, E. Breukink, B. A. van der Bent, and B. de Kruijff.** 2002. Clavanin permeabilizes target membranes via two distinctly different pH-dependent mechanisms. Biochemistry **41:**7529–7539.
27. **Wiedemann, I., et al.** 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276:**1772–1779.
28. **Wiedemann, I., et al.** 2006. Lipid II-based antimicrobial activity of the lantibiotic plantaricin C. Appl. Environ. Microbiol. **72:**2809–2814.
29. **Willey, J. M., and W. A. van der Donk.** 2007. Lantibiotics: peptides of diverse structure and function. Annu. Rev. Microbiol. **61:**477–501.