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Surface Glycosaminoglycans Protect Eukaryotic Cells against Membrane-Driven Peptide Bacteriocins

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Enzymatic elimination of surface glycosaminoglycans or inhibition of their sulfation provokes sensitizing of HT-29 and HeLa cells toward the peptide bacteriocins nisin A, plantaricin C, and pediocin PA-1/AcH. The effect can be partially reversed by heparin, which also lowers the susceptibility of *Lactococcus lactis* to nisin A. These data indicate that the negative charge of the glycosaminoglycan sulfate residues binds the positively charged bacteriocins, thus protecting eukaryotic cells from plasma membrane damage.

Bacteriocins are antimicrobial proteins produced by bacteria (1). Special attention has been devoted to peptides of between 20 and 40 amino acids produced by lactic acid bacteria (LAB) because they are usually resistant to boiling and extreme pH levels, which facilitates their use as preservatives in fermented foods (2). These peptides are divided into two major groups; class I is composed of molecules containing posttranslationally dehydrated serine and threonine residues and thioether linkages of these with neighboring cysteines to form lanthionine and 3-methyl-lanthionine, respectively, which is why they are termed "lantibiotics" (3, 4). Class II bacteriocins are composed of unmodified amino acids (2). These two classes are further subdivided depending on their secondary structure, the number of peptides that form the antimicrobial, and the spectrum of susceptible bacteria. These bactericidal peptides usually insert into the plasma membrane, polymerize there, and produce a pore, thus abolishing the membrane potential and inducing cytoplasmic solute leakage (5). These effects may be preceded by the recognition of membrane components, such as lipids I to IV, which results in the interruption of cell wall formation as well (4, 6, 7).

LAB bacteriocins are consumed with fermented foods because the microbial starters produce them in the food matrix. Based on this, nisin A and pediocin PA-1/AcH have been authorized for use as food preservatives (8). Moreover, LAB are part of the autochthonous microbiota and are believed to produce bacteriocins *in situ*. However, there have been no reports on human or animal toxicity attributable to bacteriocins, in spite of the fact that the plasma membrane is an essential part of the eukaryotic cell. The lack of toxicity of LAB bacteriocins might be due to their susceptibility to digestive proteinases or to adsorption to food components. However, nisin has been intraperitoneally injected to combat experimental infections in mice with no secondary effects (9, 10). This suggests that peptide bacteriocins are unable to open pores in the eukaryotic cell membrane. Alternatively, they might not be able to reach the membrane because the eukaryotic glycocalyx may act as a barrier, as do the lipopolysaccharides of Gram-negative bacteria (11).

Proteoglycans are part of the eukaryotic glycocalyx. They are composed of a protein stem and sulfated polysaccharide branches, called glycosaminoglycans (GAGs), that give a net negative charge to the polymer (12) and might help in blocking bacteriocins, most of which are positively charged.

Testing of this hypothesis was done by eliminating the GAG layer from the surface of cell cultures and questioning whether it made the component cells susceptible to representative peptide bacteriocins. We used nisin A (a linear lantibiotic that needs an energized bacterial plasma membrane for efficient activity) (13), plantaricin C (a globular lantibiotic that does not need a potential across the membrane) (14, 15), and pediocin PA-1/AcH (a representative of a series of class II bacteriocins that present a C-terminal moiety that specifically recognizes susceptible bacteria) (16). In this article, the results obtained from those experiments are reported.

Lactobacillus plantarum LL441 was used for plantaricin C production, using essentially the procedure described by González et al. (17). Nisin A and pediocin PA-1/AcH were obtained from Sigma. The HeLa (ATCC CCL-2) and HT-29 (ATCC HTB-38) cell lines were used for bacteriocin activity testing and were propagated according to the instructions of the supplier.

Bacteriocin activity on confluent HT-29 cell cultures was tested by the addition of each bacteriocin at 1 μg/ml (final concentration) and incubation for 24 h. No morphological changes were observed in the cells (Fig. 1A, compare photograph a with photographs e, i, and m). Similar results were obtained when the bacteriocins were used at 10 μg/ml (data not shown). Correspondingly, no viability losses were perceived after trypan blue staining of the cultures (Fig. 2B, dark-gray histograms).

Incubation of HeLa cell cultures with the bacteriocins produced similar results (Fig. 1B, compare photographs a and e for nisin A action), thus indicating that the resistance observed was not a peculiarity of HT-29 cells. Similar results were obtained for

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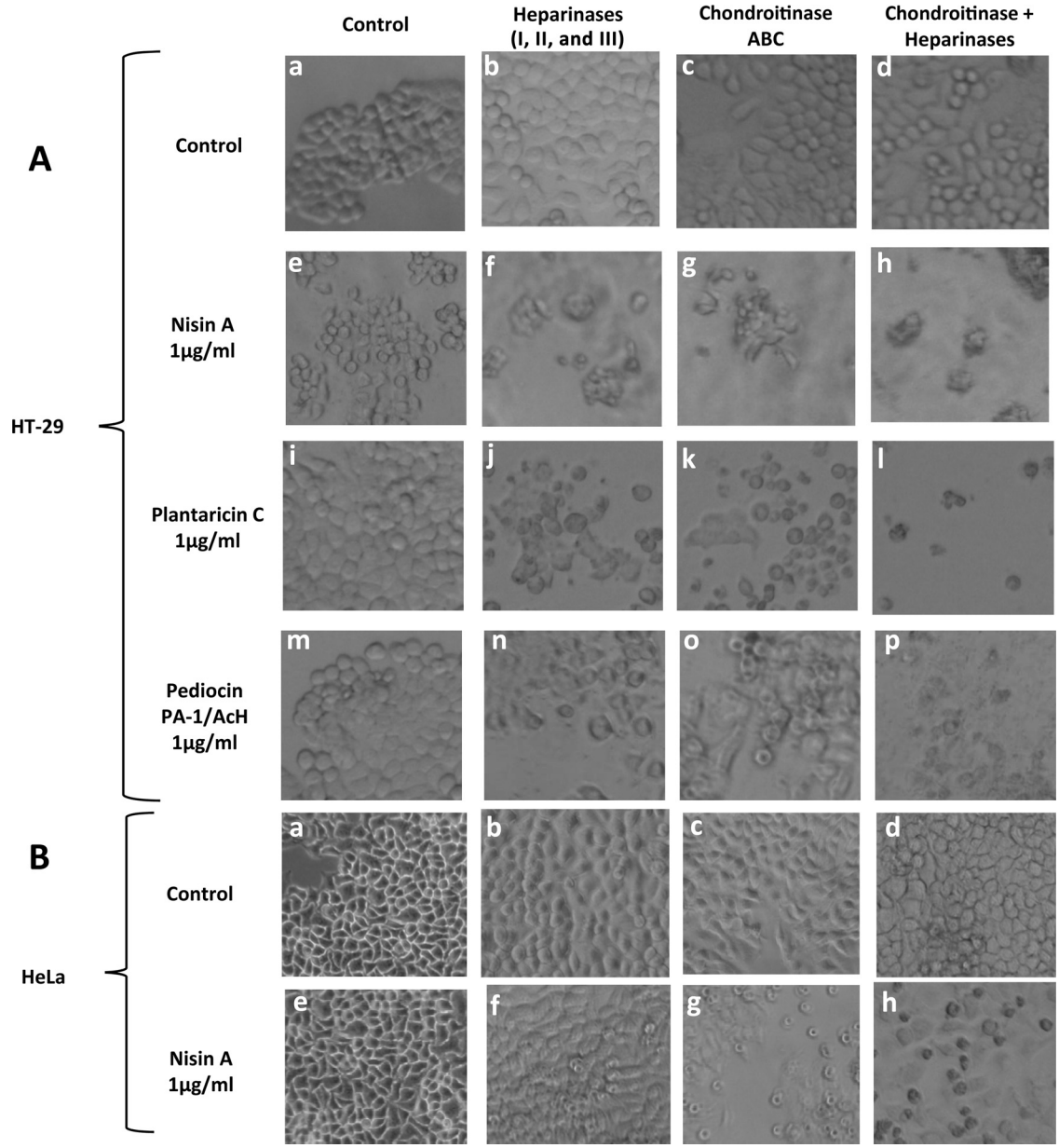

FIG 1 Appearance of HT-29 (A) and HeLa (B) cell cultures after exposure to bacteriocins, GAG-degrading enzymes, or both. Only enzyme treatments combined with bacteriocins resulted in changes in the concentrations and morphologies of the cultured cells. Representative images of results obtained from at least 3 independent experiments are shown.

plantaricin C- and pediocin PA-1/AcH-treated cultures (data not shown).

Hydrolysis of heparan sulfate or chondroitin sulfate from HT-29 and HeLa cells was achieved by overnight incubation at 37°C with a mix of 500 mU/ml each of heparinases I, II, and III (Sigma) or 250 mU/ml chondroitinase ABC (Sigma), respectively (final concentrations). Elimination of the two GAG types was achieved by successive incubation of the cell cultures with the enzymes, with an intermediate washing in phosphate-buffered saline buffer. No morphological or viability alterations were observed with respect to a control culture (Fig. 1A and B for HT-29 and HeLa cells, respectively; compare photographs a with photographs b, c, and d in each case).

The incubation of GAG-deprived HT-29 cultures with any of the three bacteriocins resulted in evident changes in cell morphology and abundance (Fig. 1A, photographs e to h for nisin A, i to l for plantaricin C, and m to p for pediocin PA-1/AcH). Similar outcomes were obtained with the HeLa cultures (Fig. 1B, photographs e to h illustrate the effect of nisin A on HeLa cells; similar pictures were obtained for the other two bacteriocins). These alterations were especially noticeable in the cultures treated with both the heparinases and chondroitinase. These data indicate that GAGs effectively protect eukaryotic cells from bacteriocin attack and that this protection is dependent on heparan and chondroitin sulfate.

To confirm the role of the GAGs in protection against a bacteriocin attack, we inhibited the last step of their biosynthesis,

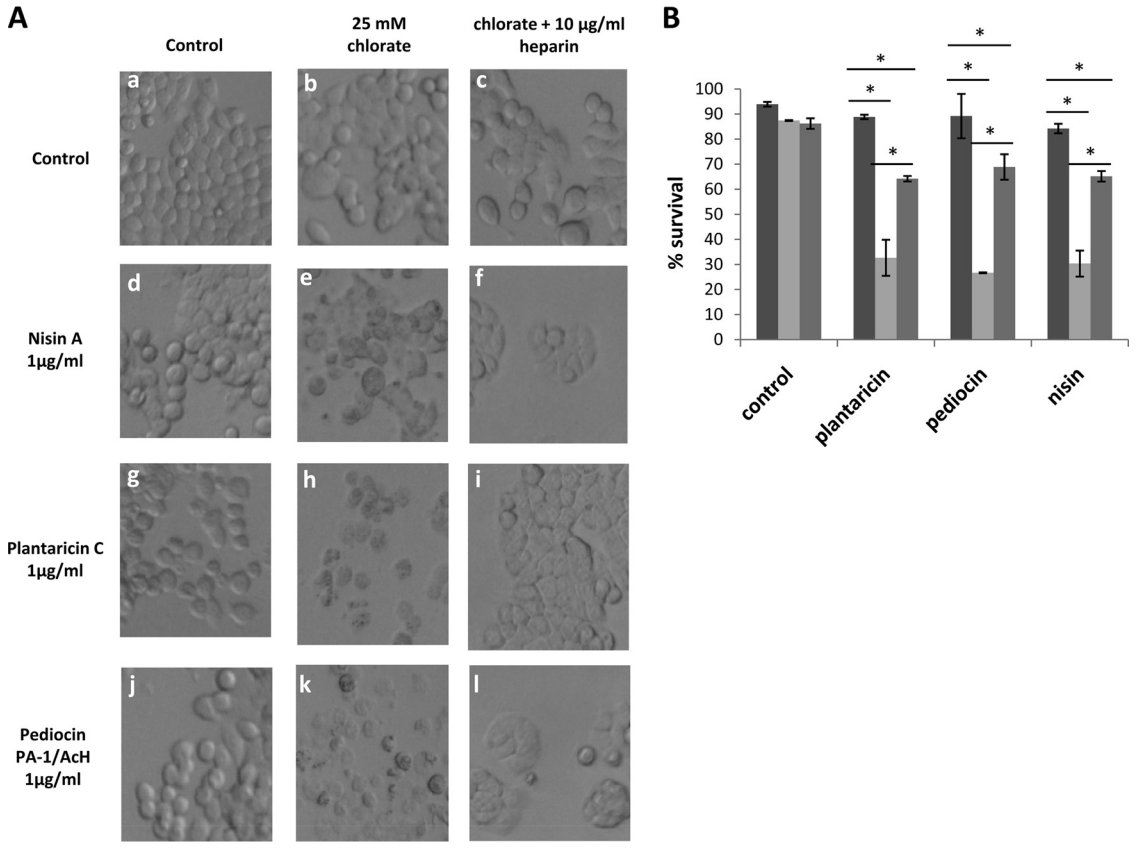

FIG 2 Effect of the bacteriocins on HT-29 cell cultures treated with sodium chlorate and protection by heparin. (A) Microscopic appearances of the cultures after the treatments indicated to the left of and above the photographs. (B) Cell viability as determined by trypan blue permeability of control cultures and replicas incubated for 24 h with each of the three bacteriocins. Dark gray, untreated cells; light gray, chlorate-treated cultures; medium gray, cultures with chlorate and heparin added simultaneously (*, $P < 0.05$). The data shown are results from at least 3 independent experiments and are represented as means ± standard error or are representative images.

namely, heteropolysaccharide sulfation, through incubation of the cell cultures in 25 mM sodium chlorate (Sigma)-containing medium for 24 h (18, 19). The chlorate did not inhibit culture growth (Fig. 2A, photographs a and b, and Fig. 2B, control). The abolition of GAG biosynthesis resulted in cultures that were susceptible to nisin A, plantaricin C, and pediocin PA-1/AcH (Fig. 2A, photographs e, h, and k, respectively, and Fig. 2B, light gray).

Finally, we found that the simultaneous addition of any bacteriocin and heparin partially reversed the susceptibilities of the chlorate-treated cultures, as determined by their appearance (Fig. 2A, photographs f, i, and l for nisin A, plantaricin C, and pediocin PA-1/AcH, respectively) and the impermeability of the cells to trypan blue (Fig. 2B, medium gray).

Final confirmation of the role of GAGs in cell protection was obtained by observation of the reduced susceptibility of *Lactococcus lactis* IL1403, a strain frequently used to titrate nisin A, to this bacteriocin. In Fig. 3, it can be observed that the preincubation of exponential-phase culture-derived *L. lactis* resting cells with 10 µg/ml heparin for 30 min prior to the addition of nisin A produced an increase in their viabilities, as observed for chlorate-treated eukaryotic cell cultures.

The ever-growing problem of antibiotic resistance is promoting the search for alternative strategies for fighting infectious diseases. One of the most promising are bacteriocins (20, 21). Most

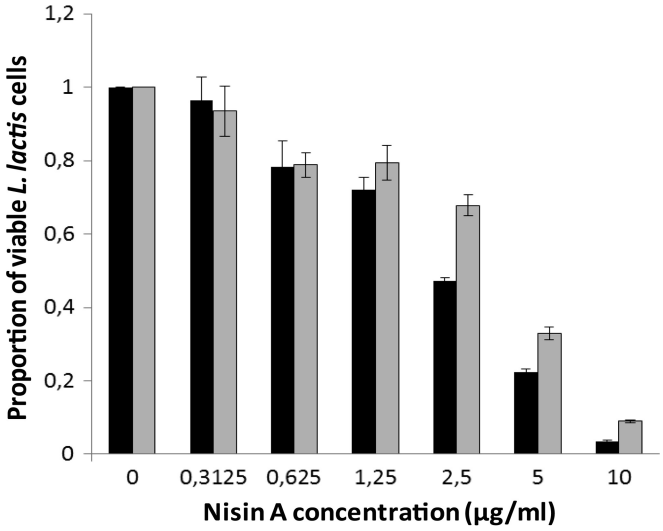

FIG 3 Survival of *Lactococcus lactis* IL1403 exponential-phase cell suspensions treated with increasing concentrations of nisin A (black) and nisin preincubated with heparin for 30 min before addition to the cell suspensions (gray). Significant differences ($P < 0.05$) were obtained only for the cultures that received the mix of nisin plus heparin from the concentration of 1.25 µg/ml nisin A and upward. The data shown are results from at least three independent experiments and are represented as means ± standard error.

are bactericidal and active at low concentrations; resistance generation is very scarce, and no adverse effects are associated with their administration (11, 13). This is surprising, since one of their targets is the plasma membrane, which is the most external structure of animal cells and, consequently, readily accessible.

The lack of toxicity of peptide bacteriocins in eukaryotic cells may be due to (i) the inability to open pores in the cell membrane due to the rigidity conferred by cholesterol (22), (ii) the lack of docking molecules (6), and (iii) the presence of extracellular barriers that would prevent the interaction of the bacteriocins with the cell membrane. However, many proteins make pores in the eukaryotic membrane, including amphipathic peptides, such as the modulins (23), defensins, cathelicidins (24), and even plantaricin A, a bacteriocin produced by *Lactobacillus plantarum* (25). Furthermore, several bacteriocins produce pores in prokaryotic membranes and liposomes in the absence of docking molecules (14, 26). Finally, the glycocalyx barrier hypothesis might be fulfilled by proteoglycans, which form a net on most tissue surfaces (27) that might prevent the penetration of bacteriocins toward the underlying cell membrane. In addition, their component GAGs are negatively charged, thus suggesting that they might bind the positively charged amino acids typical of bacteriocins.

The work presented here indicates that none of the three bacteriocins used, chosen as representative of three peptide bacteriocin classes (21), exerted significant effects on either of two eukaryotic cell types, selected as representative of the epithelial surfaces (intestinal and genitourinary) that are most commonly in contact with bacteriocins. Enzymatic elimination of heparan and/or chondroitin sulfate or inhibition of their sulfation resulted in the susceptibility of both eukaryotic cell types, whose growth became restricted and showed altered morphology. This was observed for the three bacteriocins, thus suggesting that proteoglycans mediated the resistance of intact eukaryotic cell cultures to all of them and that the sulfate component of their GAGs is responsible for the resistance shown by untreated cultures. Furthermore, the inhibitory effect of nisin A was partly reversed by the addition of heparin, which is a sulfated GAG, and this was observed with the eukaryotic cell and *L. lactis*-susceptible cultures, thus suggesting a charge complementarity interaction with the positively charged bacteriocins.

In conclusion, the resistance of eukaryotic cells to peptide bacteriocins appears to be due to binding of the sulfate groups of surface proteoglycans to positively charged bacteriocin amino acids, which prevents their interaction with the negatively charged membrane phospholipids.

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