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Complementary and Overlapping Selectivity of the Two-Peptide Bacteriocins Plantaricin EF and JK

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Plantaricin EF and JK are both two-peptide bacteriocins produced by Lactobacillus plantarum C11. The mechanism of plantaricin EF and JK action was studied on L. plantarum 965 cells. Both plantaricins form pores in the membranes of target cells and dissipate the transmembrane electrical potential (Δφ) and pH gradient (ΔpH). The plantaricin EF pores efficiently conduct small monovalent cations, but conductivity for anions is low or absent. Plantaricin JK pores show high conductivity for specific anions but low conductivity for cations. These data indicate that L. plantarum C11 produces bacteriocins with complementary ion selectivity, thereby ensuring efficient killing of target bacteria.

Many lactic acid bacteria produce bacteriocins. Bacteriocins are peptides or proteins that kill bacteria that are related to the producer strain. Bacteriocins are useful to their producer by killing bacteria that compete for the same niche. Plantaricin EF (PlnEF) and plantaricin JK (PlnJK) are both two-peptide bacteriocins that belong to the large group of small, heat-stable nonantibiotics termed class II bacteriocins (10).

Lactobacillus plantarum C11 secretes plantaricin A (PlnA), a small peptide that consists of 26 amino acids and that exhibits both bactericidal (7) and pheromone activity (4, 7). An all-D-amino-acid PlnA was found to be as bactericidal as an all-L-amino-acid PlnA. In contrast, the pheromone activity was found to be stereospecific and only observed with PlnA composed of L-amino acids (7). Therefore, the antimicrobial activity of PlnA appears not to involve chiral interactions and seems to depend only on the strong amphiphilic α-helical structure of this peptide. PlnA induces the transcription of five operons, i.e., plnABCD, plnEFI, plnJKLR, plnMNOP, and plnGHSTUV (5). The first operon contains the structural gene for PlnA itself, plnB, plnC, and plnD encode proteins that are involved in signal transduction, i.e., a two-component regulatory system that consists of the membrane-associated histidine protein kinase, PlnB, and two response regulators, PlnC and PlnD. PlnA is thought to interact with PlnB to trigger expression of the other structural genes (3, 6). The plnEFI and plnJKLR operons encode two two-peptide bacteriocins, PlnEF and PlnJK. PlnI, PlnL, PlnM, and PlnP are thought to confer immunity to the bacteriocin-producing cells (5). Based on sequence similarity, plnGH is thought to encode subunits of an ABC transporter that secretes and processes the bacteriocin precursors. The functions of PlnO (a 399-amino-acid [aa] hydrophobic peptide), PlnN (a bacteriocin-like peptide), PlnR (a 50-aa hydrophobic, cationic peptide), and PlnSTUV (hydrophobic peptides of 99, 140, 222, and 44 aa) are not known.

PlnE, PlnF, PlnJ, and PlnK are cationic peptides that consist of 33, 34, 25, and 32 amino acids and have molecular weights of 3,703, 3,545, 2,929, and 3,503, respectively (5). These peptides have the propensity to form an amphiphilic α-helical structure in a membrane-mimicking environment (8). The antimicrobial activity of PlnF is enhanced more than 1,000-fold by the equimolar presence of PlnE and vice versa. Likewise, PlnJ and PlnK are efficient antimicrobials when present together. Strikingly, none of the other combinations of these four peptides enhanced the antimicrobial activity (2, 8). The amphiphilic structure of these peptides is believed to play a role in pore formation (8, 9). The complementary peptides (PlnEF or PlnJK) interact, because the simultaneous addition of both of the peptides synergistically promoted the formation of their α-helical structure in the presence of dioleoylphosphatidylglycerol liposomes (8). In the present study, we show that PlnEF and PlnJK form pores in the target membranes that differ in ion selectivity. These results might explain why some bacteriocins are more selective to PlnEF, while others are more sensitive to PlnJK (2). The combined and complementary action of these bacteriocins warrants efficient killing of target cells.

MATERIALS AND METHODS

Materials. 86Rb⁺ (10 mCi/mg), [14C]choline⁺ (55 mCi/mmol), [14C]glutamic acid (251 mCi/mmol), and [32P] (3,000 Ci/mmol) were obtained from Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom. The separated components of PlnEF and PlnJK were synthesized and purified as described previously (8). Peptides were suspended in a solution containing 40% (vol/vol) 2-propanol, 0.1% (vol/vol) trifluoroacetic acid (TFA), and 59.9% (vol/vol) H2O. This mixture without peptide was used in control experiments and is indicated as “solvent.” The complementary components were added together in a ratio of 1:1 to give either PlnEF or PlnJK. Gramicidin A (a mixture of around 80% gramicidin A, 15% gramicidin B and 5% gramicidin C) was obtained from Sigma.

Bacterial strains and culture conditions. Lactobacillus plantarum 965 was grown at 30°C in MRS (3a) supplemented with 0.1% (vol/vol) Tween 80, but without sodium acetate and triammonium citrate. Lactose was replaced by 1% (wt/vol) glucose. In the case of phosphate efflux experiments, cells were grown in MRS broth without phosphate (20 experiments) or with 27 g of K2HPO4 per ml (unlabelled P, experiments). Cells grown up to the exponential growth phase were harvested by centrifugation (Eppendorf centrifuge, 2 min at 6,000 rpm), washed twice at 4°C, and used directly.

Uptake and efflux measurements. L. plantarum 965 cells were suspended in the buffers indicated in the figure legends, and uptake of radiolabelled compounds was monitored after energization with 0.5% (wt/vol) glucose. At indicated times, either the solvent, ionophores, or plantaricins (0.1 to 0.3 volume%) were added. In the case of choline efflux measurements, cells (500 μg of protein) were suspended in 30 μl of M17 broth (Difco) without glucose and loaded with 3.6 μCi of [14C]choline by overnight incubation at 4°C. Subsequently, cells were diluted...
in 4.2 ml of 50 mM sodium phosphate (pH 7.0) in the presence or absence of solvent, valinomycin, and nigericin or plantaricins. Samples were applied to 45-μm-pore-size cellulose nitrate filters and washed twice with ice-cold 50 mM sodium phosphate (pH 7.0) (choline efflux) or 100 mM LiCl. The radioactivity that was retained on the filter was measured by liquid scintillation counting in a Packard Tri-Carb 460 CD counter (Packard Instruments Corp.).

To measure efflux of unlabelled phosphate, cells (1.3 mg of protein/ml) suspended in 50 mM NaOH Mes [2-(N-morpholino)ethanesulfonic acid, pH 7.0] were incubated with solvent; nisin (1.4 μM); valinomycin and nigericin (250 mM each); the individual PlnE (1.4 μM), PlnF (1.4 μM), PlnK (2.8 μM) peptides; PlnEF (0.7 μM each peptide); or PlnJK (1.4 μM each peptide). To avoid cell lysis and/or continued efflux during centrifugation, samples were transferred to the top of a 20% (wt/vol) sucrose layer and directly centrifuged (2 min at 8,000 rpm in an Eppendorf centrifuge). The upper layer, containing the released Pi, was dried, subjected to destruction (30 min, 180°C), and the radioactivity that was retained on the filter was measured by liquid scintillation counting in a Packard Tri-Carb 460 CD counter (Packard Instruments Corp.).

Proton motive force measurements. The transmembrane electrical potential, Δψ, was monitored by means of Disc(5) fluorescence (excitation wavelength, 643 nm; emission wavelength, 666 nm; slit width excitation and emission, 10 nm) (21). The intracellular pH of cells was measured by using 2′,7′-bis-(2-carboxyethyl)-(and-6)-carboxyfluorescein (BCECF) as a pH-sensitive dye (excitation, 443 nm; emission wavelength, 525 nm; slit width excitation and emission, 10 nm) (13). The data were corrected for the PlnJK-induced BCECF efflux by subtraction of the PlnJK-induced BCECF fluorescence (Fig. 3, arrow 1). The latter is due to the addition of valinomycin (250 nM) (arrow 1), PlnEF (60 nM each peptide) (arrow 2), and PlnJK (100 nM each peptide) (arrow 3). a.u., arbitrary units.

**RESULTS**

PlnEF and PlnJK dissipate Δψ. The capacity of PlnEF and PlnJK to dissipate the Δψ in the sensitive strain *L. plantarum* 965 was determined. Cells were suspended in 50 mM sodium phosphate, and Δψ was generated by the addition of the potassium ionophore valinomycin. PlnEF (Fig. 1A) and PlnJK (Fig. 1B) both efficiently dissipated the Δψ. PlnEF appeared more efficient than PlnJK. The individual components had little (PlnJ, PlnK, and PlnF) or hardly detectable (PlnE) activity (data not shown).

In order to study the effect of pH on the PlnEF- and PlnJK-induced dissipation of the Δψ, the time needed to recover 50% of the Δψ-induced Disc(5) fluorescence change was measured. Both plantaricins showed a marked pH optimum around pH 6 to 6.5 (data not shown). These data demonstrate that both PlnJK and PlnEF are capable of dissipating the Δψ of target cells.

PlnJK and PlnEF dissipate ΔpH. Intracellular BCECF can be used to monitor the intracellular pH and transmembrane pH gradient (ΔpH), provided that a correction is made for the fluorescence changes due to extrusion or loss of BCECF from the cell (13, 14). Since the addition of PlnJK caused a consider-
that in contrast to PlnEF, PlnJK is able to allow BCECF efflux from the cells.

To investigate whether other anions can pass through the PlnJK pores, uptake and efflux of glutamate were measured. Although the exact mechanism of glutamate uptake has not been studied in *L. plantarum*, it is likely mediated by a system that resembles the ATP-driven, unidirectional system of *Lactococcus lactis*. When cells, after energization, were first allowed to accumulate [14C]glutamate, addition of PlnJK caused a rapid and complete release of the glutamate from the cells (Fig. 4). In contrast, PlnEF and PlnJ caused a slow release of the glutamate, while the other individual components caused no release at all. Glutamate uptake was arrested by the addition of the ionophores valinomycin and nigericin, but the accumulated glutamate was retained by the cell, analogous to previous studies with *L. lactis* (19). This indicates that indeed in *L. plantarum*, glutamate uptake takes place via a unidirectional ATP-driven mechanism. These data demonstrate that PlnJK efficiently conducts efflux of glutamate, whereas PlnEF (and PlnJ) is poorly active.

Since PlnJK caused the efflux of the anions BCECF and glutamate, we also studied whether it can cause the release of cellular phosphate. Uptake of phosphate by *L. lactis* occurs via an ATP-driven, unidirectional system (18), and a similar mechanism is anticipated in *L. plantarum*. Cells that were energized with glucose rapidly accumulated the externally added 33Pi, whereas no uptake was observed when the cells were first pretreated with PlnEF or PlnJK (see Fig. 5). On the other hand, once the 33Pi had been accumulated by the cells, addition of PlnEF, PlnJK, or the combination of valinomycin and nigericin did not induce release of the phosphate. In contrast, release was observed after the addition of nisin (Fig. 5). A colorimetric analysis of the release of cellular phosphate using a centrifugation assay to separate the cells from the suspension medium also demonstrated considerable release of phosphate only with nisin and not with PlnEF or PlnJK (data not shown). These data suggest that neither PlnEF nor PlnJK is able to conduct phosphate.

**PlnEF causes efflux of cations.** Next, the activity of the plantaricins to elicit cation release was investigated. For this purpose, cells were loaded with [14C]choline by overnight incubation. [14C]choline-loaded cells were subsequently challenged with PlnEF or PlnJK. PlnEF appeared to be more efficient in inducing choline release than PlnJK (data not shown). However, both were more effective than single peptides and the combination of valinomycin and nigericin (data not shown). In a control experiment, no plantaricin-induced Pi efflux was observed, precluding plantaricin-induced lysis. In contrast to [14C]choline, 86Rb+ ions readily accumulated in glucose-energized cells (Fig. 6). PlnEF elicited the rapid release of accumulated 86Rb+, while PlnJK blocked further uptake and caused only a marginal loss of accumulated 86Rb+. Only a slight inhibition of uptake was observed with the indi-
dissipation has also been observed for the ionophore gramicidin A (16). Gramicidin A conducts small monovalent cations, including protons (17). We found that PlnEF has high conductivity for specific anions and a low conductivity for cations. Future structural analyses may reveal the molecular bases for the cation and anion selectivity of PlnEF and PlnJK. The combined, complementary activity of PlnEF and PlnJK ensures efficient bactericidal activity.

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REFERENCES


FIG. 6. PlnEF and PlnJK inhibit 86Rb+ uptake. Cells (112 µg of protein/ml) suspended in 50 mM sodium phosphate (pH 7.0) were energized with 0.5% (wt/vol) glucose and incubated with 1.9 µM 86Rb+. At the arrow, the cells were supplemented with solvent (●), PlnE (■) (60 nM), PlnF (●) (60 nM), PlnEF (▲) (60 nM each peptide), or PlnJK (○) (120 nM each peptide).

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DISCUSSION

This study indicates that two bacteriocins, PlnEF and PlnJK, both produced by L. plantarum C11, form pores in the cytoplasmic membranes of the target cells. Both plantaricins dissipate the ΔpH and Δψ. PlnEF dissipates the Δψ more rapidly than PlnJK. In contrast, PlnJK dissipates the ΔpH more rapidly than PlnEF. In view of the observed ion selectivity, the immediate dissipation of the Δψ by PlnJK might be due to influx of hydroxyl ions. Unlike PlnJK, PlnEF first causes an increase in ΔpH before the entire ΔpH collapses. This increase in ΔpH is probably due to an enhanced proton extrusion after the dissipation of the Δψ, in analogy to the effect of valinomycin on Δψ. A temporary increase in ΔpH followed by dissipation has also been observed for the ionophore gramicidin A’ (16). Gramicidin A conducts small monovalent cations, including protons (17). We found that PlnEF has high conductivity for monovalent cations: protons and rubidium and cho- line ions. PlnJK on the other hand efficiently conducts anions, such as glutamate and BCECF. Strikingly, the C terminus of PlnF (→RAIRR) and the N terminus of PlnK (RRSRK→) are both strongly cationic. It is tempting to speculate that these sequences are involved in the anion selectivity of the PlnJK pore. However, both PlnEF and PlnJK were ineffective in causing phosphate efflux.

PlnEF dissipates Δψ more efficiently than PlnJK. This difference is presumably caused by the higher cation conductance of PlnEF compared to that of PlnJK (Fig. 6). The latter hypothesis is consistent with absence of phosphate conductance by both plantaricins and with the much higher cation concentration (50 mM) than hydroxyl ion concentration (0.1 µM) at pH 7. Both growth inhibition of L. plantarum 965 (2) and ΔpH dissipation (Fig. 2) occur more efficiently by PlnJK than by PlnEF. This suggests that Δψ dissipation more effectively causes growth inhibition than ΔpH dissipation. Δψ dissipation leads to a drop in intracellular pH and consequent inhibition of metabolism and thus of substrate-level phosphorylation which provides the cell with metabolic energy in the form of ATP. All two-peptide class II bacteriocins dissipate Δψ (1, 12, 15, 22). On the basis of the ion selectivity, two subgroups of two-peptide bacteriocins can now be identified: (i) monovalent cation-conducting systems such as lactococcin G (15, 16) and PlnEF (lactococcin G does, however, not conduct protons) and (ii) bacteriocins with a preference for anions (i.e., PlnJK and possibly acidocin J1132) (22). In contrast to the bacteriocins described above, lactacin F seems to lead to efflux of both potassium ions and phosphate (1).

In conclusion, L. plantarum C11 produces three antimicrobial peptide systems, i.e., the bacteriocin-like pheromone PlnA, the cation-conducting PlnEF, and PlnJK, which exhibits a high conductivity for specific anions and a low conductivity for cations. Future structural analyses may reveal the molecular bases for the cation and anion selectivity of PlnEF and PlnJK. The combined, complementary activity of PlnEF and PlnJK ensures efficient bactericidal activity.

FIG. 6. PlnEF and PlnJK inhibit 86Rb+ uptake. Cells (112 µg of protein/ml) suspended in 50 mM sodium phosphate (pH 7.0) were energized with 0.5% (wt/vol) glucose and incubated with 1.9 µM 86Rb+. At the arrow, the cells were supplemented with solvent (●), PlnE (■) (60 nM), PlnF (●) (60 nM), PlnEF (▲) (60 nM each peptide), or PlnJK (○) (120 nM each peptide).


