Enterocin P Causes Potassium Ion Efflux from Enterococcus faecium T136 Cells

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Enterocin P is a bacteriocin produced by Enterococcus faecium P13. We studied the mechanism of its bactericidal action using enterocin-P-sensitive E. faecium T136 cells. The bacteriocin is incapable of dissipating the transmembrane pH gradient. On the other hand, depending on the buffer used, enterocin P dissipates the transmembrane potential. Enterocin P efficiently elicits efflux of potassium ions, but not of intracellularly accumulated anions like phosphate and glutamate. Taken together, these data demonstrate that enterocin P forms specific, potassium ion-conducting pores in the cytoplasmic membrane of target cells.

Antimicrobial peptides occur in a wide range of organisms, including bacteria, plants, and animals (10, 21). Many lactic acid bacteria produce a special kind of antimicrobial peptides or protein, the so-called bacteriocins (9, 20). Bacteriocins are peptides or proteins that kill bacteria related to the producer strain. In the struggle for a niche and nutrients, bacteriocins are useful to their producers by killing competing bacteria. Bacteriocins have been classified into three groups (19, 20): (i) lantibiotics, which contain posttranslationally modified amino acids, such as lanthionine and β-methyl-lanthionine, (ii) small (<10 kDa), heat-stable peptides without posttranslationally modified amino acids, which are subdivided into four subgroups (IIa, peptides with the N-terminal consensus sequence YGNGVXC, strongly active against Listeria spp.; IIb, two-peptide systems; IIc, sec-dependent bacteriocins; IIId, class II bacteriocins not included in the previous groups), and (iii) large (>30 kDa), heat-labile proteins. Enterocin P is a bacteriocin composed of 44 amino acids produced by Enterococcus faecium P13 (5) and other related strains (11). Since it is synthesized with a cleavable signal sequence, it may be secreted against food-borne gram-positive pathogenic bacteria, including Staphylococcus aureus, Clostridium perfringens, Clostridium botulinum, and Listeria monocytogenes (5). Here, we studied the mode of bacteriocidal activity exerted by enterocin P on enterocin-P-sensitive E. faecium T136 cells.

MATERIALS AND METHODS

Materials. 86Rb+ (10 mCi/mg), [14C]glutamic acid (260 mCi/mmol), and [32P]Pi (3,000 Ci/mmol) were obtained from Amersham, Little Chalfont, United Kingdom. Dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylglycerol (DOPG) were obtained from Avanti Polar Lipids. Nisin was a gift from Aplin & Barrett. The fluorescent probes 3-benzene-1,4,10,13-tetraoxa-7,16-diazacyclododecane-7,16-diybis(5-methoxy-6,2-benzofurandiyll) (PBFI), 3,3'-dipropylthiadicarbocyanine iodide [DiSC_5(5)], and 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF) were obtained from Molecular Probes, Eugene, Oreg.

Strains and culture conditions. Enterocin P-sensitive (T136s) (5) and enterocin P-resistant (T136r) strains of E. faecium T136 were used in addition to the producer strain E. faecium P13. T136r cells were isolated after selection by treating 106 CFU of T136 cells/ml with 10,000 bacteriocin units (BU)/ml of enterocin P for 24 h. E. faecium T136r was not at all inhibited by an enterocin P sample that showed activity of 7,500 BU/ml against E. faecium T136. The enterocin P resistance of E. faecium T136r remained stable after culturing in the absence of enterocin P. Both enterocin P-resistant (T136r) and enterocin P-sensitive (T136s) E. faecium T136 cells produce the enterocins A and B (4). Cells were grown in MRS broth (Oxoid) at 30°C and harvested in the logarithmic growth phase.

Enterocin P purification and antimicrobial activity assays. Enterocin P was purified as described previously (5). The bacteriocin was dissolved in 60% (vol/vol) isopropanol and 0.1% (vol/vol) trihydroxyacetic acid and stored at -20°C. An equal volume of the solvent without bacteriocin was used in control experiments. Bacteriocin activity was measured using a microtiter plate assay system (12). In brief, the optical density at 660 nm of microwell cultures exposed to a range of bacteriocin concentrations was measured.

Proton motive force measurements. The transmembrane pH gradient (ΔpH) was measured by monitoring the fluorescence of the pH-sensitive fluorescent probe BCECF (excitation wavelength, 502 nm, and slit width, 5 nm; emission wavelength, 525 nm, and slit width, 15 nm) (17). Cells were loaded with BCECF by incubating 20 μl of 50 mM KP, cell suspension with 1 to 3 μl of 10 mM BICECF and 2 to 2.5 μl of 0.5 N HCl for 5 min. The loading was followed by four rapid washes (Eppendorf centrifuge, 2 min at 6,000 rpm). The transmembrane electrical potential (ΔΨ) was recorded by measuring the fluorescence of 0.5 mM DiSC_5(5) (excitation wavelength, 643 nm, and slit width, 10 nm; emission wavelength, 666 nm, and slit width, 10 nm) (22).

Uptake and efflux measurements. E. faecium cells were harvested in the logarithmic growth phase, washed, and suspended at 137.5 μg of protein/ml in the buffers (1 to 2 ml) described in the figure legends. Uptake of radiolabeled compounds was monitored after the energization of the cells with 0.5% (wt/vol) glucose. After 20 min, either enterocin P (60 BU/ml) or solvent was added. At intervals, samples (100 μl) were applied to 45-μm-pore-size cellulose nitrate filters (Millipore Corp.) and washed twice with 2 ml of 50 mM morpholineethanesulfonic acid (MES)-NaOH (pH 7.0) (P, efflux) or 100 mM LiCl. The radioactivity retained by the filters was measured by liquid scintillation counting in a Tri-Carb 460 CD counter (Packard Instruments Corp.).

Liposomes composed of DOPG-DOPC (1:1 [wt/wt]) and prepared by reverse-phase evaporation (23) were loaded with 86Rb+ by overnight incubation at room temperature. Enterocin P (80 BU/ml) or solvent was added and efflux was measured as described above, except that filters were washed twice with 2 ml of 50 mM NaP, pH 7.
**K⁺ flux measurements.** Flux of K⁺ was monitored by the K⁺-specific fluorescent indicator PBFI (excitation wavelength, 336 nm, and slit width, 15.0 nm; emission wavelength, 507 nm, and slit width, 8.0 nm) (13). Liposomes composed of DOPG-DOPC (1:1 [wt/wt]) were prepared by ethanol injection (1). Extraliposomal potassium was removed by centrifuging potassium-loaded liposomes for 15 min at 280,000 x g.

**Miscellaneous methods.** Protein concentration was measured by the DC Protein Assay (Bio-Rad, Hercules, Calif.). The hydrophobicity profile of enterocin P was calculated with a 19-residue window by using the hydrophobicity scale of Eisenberg (8). Experiments were performed at 30°C and repeated at least three independent times, and typical experiments are presented.

**RESULTS**

**Enterocin P-mediated ΔΨ dissipation.** The ability of enterocin P to dissipate ΔΨ was studied by two independent methods. First, ΔΨ was induced by energizing cells with glucose, followed by conversion of ΔpH into ΔΨ by the addition of the H⁺/K⁺ exchanger nigericin. Formation of ΔΨ resulted in a decrease in DiSC₃(5) fluorescence. Enterocin P dissipated ΔΨ of *E. faecium* T136s cells in 50 mM K-HEPES (pH 7.0) (Fig. 1B) at a concentration-dependent rate (data not shown). By contrast, enterocin P-resistant *E. faecium* T136r cells (Fig. 1A) appeared to be completely insensitive to enterocin P action. Secondly, ΔΨ was generated by addition of the potassium ionophore valinomycin to nonenergized cells suspended in buffers without potassium. Under these conditions, enterocin P did not cause any ΔΨ dissipation (data not shown).

**Enterocin P elicits K⁺ efflux from sensitive cells.** Since enterocin P is capable of ΔΨ dissipation, it must conduct ion or proton movements across the membrane. Therefore, we investigated the ability of enterocin P to elicit transmembrane ion movements. In previous experiments (C. Herranz, unpublished data), the bacteriocin did not dissipate the ΔpH in energized cells, indicating an absence of proton conductance. To exclude that ATP-consuming proton extrusion compensated for the

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**FIG. 1.** Enterocin P dissipates ΔΨ. Shown are enterocin P-resistant cells (T136r) (A) and enterocin P-sensitive cells (T136s) (B). To *E. faecium* cells (14 μg of protein/ml) suspended in 50 mM K-HEPES (pH 7.0), glucose (0.5%; arrows 1) and nigericin (250 nM; arrows 2) were added, resulting in a decrease in DiSC₃(5) fluorescence and thus the generation of ΔΨ. Subsequently, enterocin P (80 BU/ml; arrows 3) and nisin (6 μM; arrows 4) were added, resulting (eventually) in the recovery of DiSC₃(5) fluorescence and dissipation of ΔΨ.

**FIG. 2.** Enterocin P causes the efflux of °⁹⁹Rb⁺ from sensitive cells. Cells (137.5 μg of protein/ml) suspended in 50 mM NaPᵢ were energized with 0.5% glucose, thus allowing °⁹⁹Rb⁺ uptake. At the arrow, enterocin P (60 BU/ml) was added to *E. faecium* T136s (○), *E. faecium* P13 (▲), and *E. faecium* T136r (●) cells and solvent was added to *E. faecium* T136s (■) cells.
Synthetic PC-PG liposomes, loaded with PBFI and KPi buffer, show PBFI fluorescence, indicating the release of potassium ions. Subsequent addition of enterocin P caused a reversal of the decrease in the fluorescence due to potassium uptake (Fig. 3). Glucose to a cell suspension with external PBFI resulted in a potassium ion-specific fluorescent probe PBFI. Addition of potassium ion efflux, experiments were performed with the producer cells did not decrease at all. In order to verify the producer cells did not decrease at all. In order to verify the presence of enterocin P or only solvent. On the other hand, nisin caused a significant and rapid release of the glutamate (data not shown). Enterocin P also did not inhibit uptake of glutamate (data not shown). These results suggest that enterocin P does not form aspecific pores.

**DISCUSSION**

Here, we investigated the bactericidal action of the bacteriocin enterocin P by studying its effect on transport processes in *E. faecium* T136s cells. Enterocin P causes a rapid and drastic efflux of the intracellulary accumulated potassium ion analog 86Rb⁺ from *E. faecium* T136s cells. Efflux of potassium ions measured by the specific fluorescent probe PBFI shows a rapid release of intracellular K⁺. This enterocin P-mediated potassium ion efflux is highly specific, as under the same sets of conditions, no dissipation of the ΔΨ was observed. Moreover, the collapse of the ΔΨ occurred only under specific conditions. No enterocin P-mediated dissipation is observed for a valinomycin-induced ΔΨ in cells suspended in Na⁺ or in choline-containing buffers. This indicates that the bacteriocin conducts neither sodium or choline ion influx. Finally, enterocin P does not cause efflux of ATP (Herranz, unpublished), radiolabeled phosphate, or glutamate.

Enterocin P has no activity at all against the producer and resistant cells nor does it seem to act on synthetic liposomes. A putative immunity protein-encoding gene is present in the producer *E. faecium* P13 cells (5). One might speculate that a receptor-like factor, which is absent in synthetic liposomes, might be dysfunctional in the resistant cells. However, the bacteriocin has activity against species from a variety of genera (5), which reduces the likelihood of a required cell factor.

Strikingly, enterocin P resembles the two-component bacteriocin lactococcin G in its high capacity to conduct potassium ions whereas there is no conductance of protons. In contrast to enterocin P, lactococcin G conducts a range of monovalent cations, including sodium and chloride ions (18). The two-component lantibiotic lactacin 3147 dissipates ΔΨ only indirectly after prolonged incubation that results in a depletion of the ATP pool (16).

Various mechanisms of membrane permeabilization by antimicrobial peptides have been proposed: the wedge-like model (6), the transmembrane helical bundle of hydrophobic peptides, the thioroidal model, the in-plane diffusion model, and the carpet...
model (3). Enterocin P pores may be composed of transmembrane bundles of hydrophobic peptides. This is supported by the high ion specificity of enterocin P and by the presence of a highly hydrophobic transmembrane segment in the C-terminal part of enterocin P (Fig. 4). Since the hydrophobicity increases in the N-to-C-terminal direction, the C-terminal segment of enterocin P may possibly insert into the membrane, whereby the C-terminal histidine reaches the cytoplasm. In this respect, a C-terminal N-to-C-terminal direction, the C-terminal segment of enterocin P (Fig. 4). Since the hydrophobicity increases in the N-to-C-terminal direction, the C-terminal segment of enterocin P may possibly insert into the membrane, whereby the C-terminal histidine reaches the cytoplasm. In this respect, a C-terminal intracellular histidine has been shown to determine the activity of a potassium channel (2).

Taken together, bundles of transmembrane enterocin P peptides may form a pore that specifically conducts potassium ions. Future reconstitution of potassium ion-conducting enterocin P activity in liposomes may reveal the molecular interactions that underlie the observed high ion specificity of enterocin P pores.

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