

CLINICAL STUDY

Mode of action of lactocin 160, a bacteriocin from vaginal *Lactobacillus rhamnosus*

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Abstract

Objectives. To determine the mechanism of antimicrobial action of lactocin 160, a bacteriocin produced by the healthy vaginal strain of *Lactobacillus rhamnosus*, using an established model, with *Micrococcus luteus* ATCC 10420 as a test organism. **Methods.** Sensitivity of *M. luteus* to lactocin 160 was determined by the diffusion assay. Loss of cellular ATP in the lactocin-treated cells was elucidated using a commercially available ATP determination kit (luciferin-luciferase bioluminescence assay). Luminescence intensity as a reflection of ATP quantity was determined using a luminometer. Dissipation of membrane potential ($\Delta\psi$) was studied using fluorophore DiSC3(5) with the fluorescence spectrum sensitive to changes in $\Delta\psi$. **Results.** Lactocin 160 inhibited growth of *M. luteus* ATCC 10420 at a concentration of 5 $\mu\text{g/ml}$. There were no significant changes in the intracellular ATP level of *M. luteus* upon the addition of 20 $\mu\text{g/ml}$ of lactocin 160. However, the extracellular ATP level increased significantly. This means that the treatment of cells with lactocin 160 resulted in an efflux of ATP from inside the cells. Therefore, a partially purified lactocin 160 preparation (16 $\mu\text{g/ml}$ of the bacteriocin in the sample) killed sensitive cells and dissipated $3.12 \pm 0.36\%$ of $\Delta\psi$.

Conclusion. Lactocin 160 has a mode of action typical for bacteriocins. It disturbs the cellular membrane ($\Delta\psi$ dissipation) and induces ATP efflux, most likely because of the pore formation, which is a common mechanism of action for many bacteriocins.

Keywords: Lactobacillus, bacteriocin, ATP, membrane potential

Introduction

Bacterial vaginosis (BV) is a vaginal, multi-microbial syndrome that can be a risk factor for human immunodeficiency virus (HIV) transmission and acquisition. Several cross-sectional studies identify BV as a cofactor in the sexual transmission of HIV [1–3]. Major BV organisms directly up-regulate HIV replication [4–6]. Our *in vitro* data indicated that the most frequent BV-associated microorganisms *Gardnerella vaginalis*, *Peptostreptococcus* spp and *Prevotella bivia* stimulate the expression of HIV [5,7].

Lactobacilli in the lower genital tract of healthy females protect the vagina from the invasion of pathogens by producing antimicrobial substances, such as weak organic acids (mostly lactic acid), bacteriocins and hydrogen peroxide [8–10]. Bacteriocins are defined as proteinaceous substances that exhibit antibacterial activity. Vaginal lactobacilli produce bacteriocins effective against vaginal pathogens such as *Enterococcus* spp and *Neisseria gonorrhoeae* [11], and may have a fungistatic effect on *Candida albicans* [12]. Among 22 vaginal *Lactobacillus* strains studied by Aroutcheva et al. [9,13], 77.3% exhibited antimicrobial activity against *G. vaginalis*. One of these strains, designated *Lactobacillus rhamnosus* 160, produces a bacteriocin named lactocin 160 that inhibits growth of *G. vaginalis* [13].

Here we report for the first time, our preliminary data on the mechanism of antimicrobial action for the bacteriocin produced by the vaginal strain of *Lactobacillus*, lactocin 160, using a well-established model where *Micrococcus luteus* ATCC 10420 is a test organism [14].

Materials and methods

Lactocin 160 purification

The bacteriocin producer *L. rhamnosus* 160 was isolated from a patient with healthy vaginal microflora. Lactocin 160 was purified following the previously described procedure [15]. Briefly, 2000 ml of the overnight culture of *L. rhamnosus* 160 grown in MRS broth (Remel, KS) anaerobically at 36°C, were concentrated by centrifugation (7000 x g, 20 min at 5°C), washed three times in 1 x PBS (pH 6.0) and transferred into 200 ml of chemically defined media (CDM) composed from substances found in vaginal secretion but without any proteins and amino acids [16]. The cells were incubated anaerobically at 37°C for 18 h with constant shaking and then removed by centrifugation at 12,000 x g for 25 min at 5°C.

The non-lactocin 160 proteins were precipitated from the CDM by 80% ammonium sulfate for 3 h at 5°C. The precipitate was removed by centrifugation at 12,000 x g for 25 min at 5°C. The supernatant containing lactocin 160 was dialyzed against deionized water, using a dialysis bag with MW CO 500 (Spectrum, CA), within 3 days. The dialyzed preparations were concentrated by lyophilization.

The 10–20% pre-cast sodium dodecyl sulfate (SDS) polyacrilamide gel (Bio-Rad, CA) electrophoresis (SDS-PAGE) was conducted to determine the presence of lactocin 160 in the sample. The SDS-PAGE was conducted according to the Bio-Rad manual at 200 V for 90 min. To visualize proteins, the gel was stained with a Bio-Rad Silver Stain according to the manufacturer's instructions.

Nisin preparation

Nisin powder, purified bacteriocin produced by *Lactococcus lactis*, was a gift from Aplin & Barrett (Beaminster, UK). Nisin is the only lactic acid bacteria-produced bacteriocin that is used for food preservation in more than fifty countries. Because it is a class Ia bacteriocin from *Lactococcus lactis*, with a well-studied mechanism of action, nisin was used as a positive control [17].

A high concentration of nisin solution can only be obtained at a low pH since this bacteriocin has the best solubility in the pH range of 1.7–3.5. To prepare a highly concentrated nisin stock solution, one gram of dry preparation of the bacteriocin was dissolved in 1 ml of commonly used "nisin diluent" consisting of 0.02 N HCl (pH 1.7). The stock solution was used to prepare working concentrations in the PBS buffer (pH 7.2). Because of the high buffer capacity of the PBS and the small amounts of nisin in final preparations, the pH of nisin working solutions was approximately 7.0.

Well diffusion assay

The stock culture of *Micrococcus luteus* ATCC 10420 used in this study was stored in a nutrient broth (Difco, MI) with 20% glycerol at –70°C. The strain was streaked on a fresh nutrient agar (Difco, MI) plate three times before it was used as working culture, which was kept at 4°C. The CDM was used as a negative control. To conduct the susceptibility test, a partially purified preparation of lactocin 160 was dissolved in PBS (pH 7.2) at 20 mg/ml.

Before pouring plates, *M. luteus* ATCC 10420 cells were seeded into the nutrient agar to make a final concentration of approximately 10⁴ CFU/ml. When the agar was polymerized in the Petri dishes, wells 6 mm in diameter were punctured in the agar. Then 80 µl of the 2-fold dilutions of lactocin 160 preparation was added into the wells. The agar plates were pre-incubated at 4°C for 6 h, so the bacteriocins could diffuse into the agar, followed by 24 h of incubation at 30°C. The observed inhibition zones indicated activity of the bacteriocins.

Protein assay

To determine protein (the bacteriocin) concentration in the partially purified preparation of lactocin 160, the RC DCTM Protein Assay kit (BioRad) was used according to the manufacturer's manual.

ATP assay

First, an ATP standard curve was obtained by following the procedure described in the manual (Technical Bulletin No. BAAB-1) included with the ATP bioluminescent assay kit (FL-AA, Sigma-Aldrich Corporation, St. Louis, MO). The total ATP level of both the normal and stressed cells was determined as described previously [18]. Briefly, 1 ml of *M. luteus* ATCC 10420 was collected by centrifugation, and the pellet was re-suspended in a 50 mM MES (2-[N-Morpholino]-ethanesulfonic acid) buffer (pH 6.5) supplemented with 0.2% glucose and 10 mM KCl to energize the cells. The cell suspension was divided into 2 aliquots. One aliquot was treated with lactocin 160, and the other (no bacteriocin added) served as the control. After 10 min of energization with glucose, the 10 mg/ml (final concentration) lactocin 160 partially purified preparation was added in one aliquot of cell suspension. At the same time, the same volume of the MES buffer was added to the control cells. For both cells with and without lactocin 160 treatment, the procedures were the same. To determine the total ATP level, at pre-determined intervals, 20 µl of the cell suspension

was quickly added to 80 μ l DMSO (dimethyl sulfoxide, Fischer Biotech, NJ) and mixed for 30 s before dilution with 4.9 ml of cold water (4°C). To measure the extracellular ATP level, 20 μ l of the same cell suspension was mixed with 80 μ l H₂O instead of DMSO. The subsequent steps were the same as those for the total ATP determination. The 100 μ l of the cells in DMSO or cells in H₂O was added to the 100 μ l ATP assay enzyme solution (containing luciferase, luciferin, MgSO₄, DTT, EDTA BSA and Tricine buffer salt) (1:25 dilution of stock) and incubated. Relative luminescence intensity (RLI) was determined instantly using a luminometer (Thermo Labsystem, Franklin, MA). RLI data were converted into ATP concentration using the ATP standard curve constructed earlier. The intracellular ATP level was calculated by subtracting the extracellular ATP level from the total ATP level.

Membrane potential ($\Delta\psi$) dissipation

The mid-log phase *M. luteus* culture was collected by centrifugation and re-suspended in 50 mM HEPES (pH 7.0). The cells were washed twice in the buffer before they were re-suspended in the buffer, which was ten times smaller in volume, to achieve OD₆₀₀ = 40. Fluorescence measurements were performed in a PerkinElmer LS-50B spectrofluorometer (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Parameters were set up as: excitation = 643 nm; emission = 666 nm; and slit width = 10 nm for both. For each measurement, 5 μ l of 2 mM DiSC3 were added to a 2 ml 50 mM K-HEPES buffer (pH 7.0) to reach a final concentration of 5 μ M. After stabilization of the baseline, 20 μ l cells were added (final OD₆₀₀ was ~0.3) followed by 20 μ l 20% glucose to energize the cells. Then 2 μ l of 5 mM nigericin (final concentration of 5 μ M) were added to convert the pH gradient. Different amounts of either nisin or lactocin were used to deplete $\Delta\psi$ after signal stabilization. Finally, 20 μ l 0.3 mM valinomycin (final concentration of 3 μ M) were added to observe complete dissipation of the $\Delta\psi$. The increase in the fluorescence intensity induced by different bacteriocins or different concentrations of the same bacteriocin was compared. Fluorescence intensity after the addition of nigericin was denoted as I_0 . Intensity following the addition of any bacteriocin was denoted as I_t and intensity after valinomycin-induced total depletion of membrane potential was I_∞ . Therefore, the $\Delta\psi$ dissipation percentage (%) can be calculated as

$$\Delta\psi \text{ dissipation \%} = (I_t - I_0) / (I_\infty - I_0) \times 100$$

Statistical analysis

The variance of the data between the replicates was less than 10%. Standard deviation was calculated for each cell population studied.

Results

Using the method developed in our laboratory, we isolated the antibacterial peptide with the molecular weight of 3.8 kDa (Figure 1). According to the SDS-PAGE, lactocin 160 was the only protein present in the preparation. However in addition to the peptide, the lactocin 160 partially purified preparation contained CDM components and possibly metabolic products of a non-proteinaceous nature.

The minimum inhibitory concentration (MIC) of the lactocin 160 partially purified preparation against *M. luteus* ATCC 10420 was 5 μ g/ml. CDM had no effect on the growth of the tested microorganism.

There were no significant changes in the intracellular ATP level of *M. luteus* upon the addition of 20 μ g/ml of lactocin 160 (partially purified preparation of 20 mg/ml of total sample, data not shown). At the same time, the extracellular ATP level increased

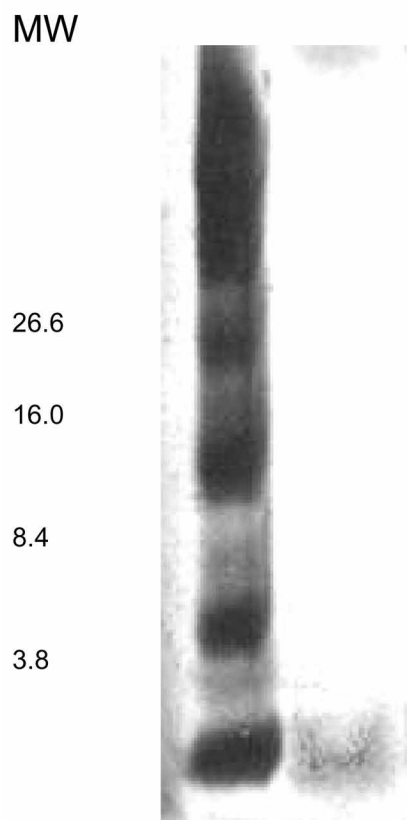


Figure 1. Lactocin 160, MW ~ 3.8 kDa.

significantly (Figure 2). This means that treatment of the cells with lactocin 160 resulted in an efflux of ATP from inside of the cells.

As shown in Table I, 8.0 mg/ml of partially purified lactocin 160 preparation (16 μg /ml of the bacteriocin in the sample) dissipated $3.12 \pm 0.36\%$ of $\Delta\psi$.

Discussion

Bacteriocins exhibit inhibitory effects against various pathogens in a manner similar to antibiotics. However, bacteriocins are distinguished from antibiotics on the basis of their synthesis, mode of action, toxicity and resistance mechanisms [15,16]. Organisms resistant to antibiotics are generally not cross-resistant with bacteriocins, and bacteriocin resistance is not always genetically determined [17].

Bacteriocins kill sensitive cells by depleting the transmembrane potential ($\Delta\psi$) and/or the pH gradient and forming pores in the membrane, resulting in the leakage of cellular materials [18].

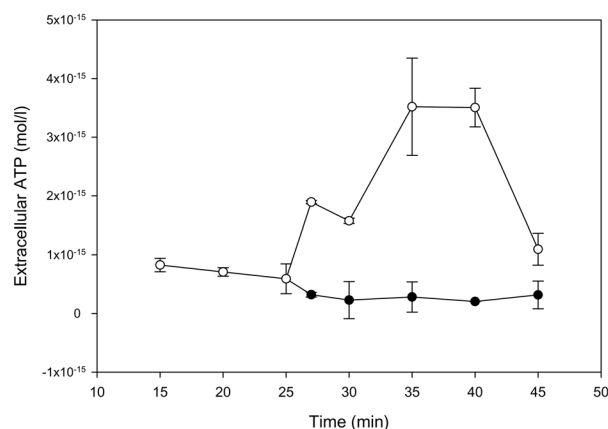


Figure 2. Influence of lactocin 160 on extracellular ATP levels in *M. luteus* ATCC 10420. The changes in extracellular ATP level induced by lactocin 160 (open circles) were compared with ATP level in non-treated cells (closed circles).

Table I. Dissipation of $\Delta\psi$ in *M. luteus* ATCC 10420 by lactocin 160.

Amount of partially purified lactocin 160 preparation		
Total sample mg/ml	Total protein $\mu\text{g}/\text{ml}$	$\Delta\psi$ dissipation %
0.4	0.8	0
2.0	4	1.83 ± 0.27
8.0	16	3.12 ± 0.36
Pure nisin (control) $\mu\text{g}/\text{ml}$		
1	3.66 ± 0.45	
5	18.18 ± 3.10	
10	42.35 ± 5.27	

Electrostatic interactions with negatively charged phosphate groups on target cell membranes contribute to the initial binding of many bacteriocins to the sensitive cell's membrane [19,20]. Other factors, such as the phospholipid composition of the target bacterial membrane and the environmental pH, influence the bacteriocin's activity [19].

In this study we elucidated the general mechanism of action of partially purified lactocin 160 from vaginal *L. rhamnosus* against *M. luteus* ATCC 10420. We intentionally studied the peptide in its partially purified form, which contains a trace of media components. Lactocin 160 is normally secreted from the strain into the vaginal environment. We speculate that the CDM, which mimics vaginal fluid, may also stabilize and protect the peptide from degradation. Therefore, it should be present in lactocin 160 topical vaginal microbicide formulation.

It was shown that lactocin 160 disturbs the cellular membrane and induces ATP efflux. Bacteriocins are known to deplete intracellular ATP as a result of pore formation [17]. Dissipation of $\Delta\psi$ is a typical characteristic of the pore-forming bacteriocin as it indicates the disturbance of the membrane's integrity by the bacteriocin molecules. In this study, we found that lactocin 160 dissipates the membrane potential of sensitive cells. Thus, dissipation of membrane potential is only part of the mode of action of lactocin 160. In addition, lactocin 160 did not affect the membrane pH gradient (ΔpH) of *M. luteus* (data not shown). This was not a surprise since some bacteriocins selectively dissipate one component of the proton motive force. Bacteriocins, such as lactococcin G and lactocin 3147 were reported to induce pores selective for K^+ ions and inorganic phosphate [21–24]. Enterocin P caused a significant reduction of membrane potential ($\Delta\psi$) and the intracellular pool of ATP from *Enterococcus faecium* T136. However, the ΔpH was not affected in the cells of *E. faecium* treated with enterocin P [21]. Therefore in addition to the membrane potential dissipation, there must be some other mechanism(s) that contribute(s) to the antimicrobial action of lactocin 160 observed in the well-diffusion assay.

Using the commonly accepted method for testing bacteriocin activity, with *M. luteus* ATCC 10420 as the test strain, we showed that a partially purified preparation of lactocin 160 has a mode of action typical for bacteriocins. The antimicrobial activity of this partially purified lactocin 160 preparation was somewhat low for *M. luteus* ATCC 10420. This is because this preparation is likely to contain media components of a non-proteinaceous nature and metabolic products that were not removed by the partial purification. Therefore we

assume that the lactocin 160, purified to homogeneity, will have a much higher activity suitable for application. However, the goal of this research was not to purify lactocin 160 to homogeneity but to obtain, for the first time, preliminary data on the possible mechanism of action of lactocin 160 isolated from a vaginal specimen dominated by *Lactobacillus*. The preliminary mode of action study was performed using the strain of *M. luteus* that is commonly accepted as a reference bacteriocin-sensitive organism. However, it is not the most lactocin 160-sensitive strain and it is not a vaginal isolate. In fact, vaginal pathogens are more sensitive to lactocin 160 than *M. luteus* (personal communication). In addition commercially available topical antimicrobials, such as 2% cleocin and 0.75% metrogel that are usually prescribed for the treatment of bacterial vaginosis and other vaginitis, release 100 mg of clindamycin (single dose) and 37.5 mg of metronidazole. These very high concentrations of antimicrobials kill not only BV-associated bacteria but also healthy vaginal lactobacilli [25,26]. At the same time, our partially purified preparation of lactocin 160 kills BV-associated microorganisms but does not kill healthy vaginal microflora, therefore it is specific in its antimicrobial action (personal communication).

The rapidly increasing prevalence of genital pathogens resistant to conventional antibiotics has prompted a search for new therapeutic agents. An antibacterial peptide produced by vaginal lactobacilli and having a mechanism of action different from conventional antibiotics can be a good choice for the replacement of antibiotics in the prophylaxis of BV. Further study of lactocin 160s mechanism of action will be performed using *G. vaginalis* as a major BV-associated microorganism and purified to homogeneity preparation of the bacteriocin.

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