MODE OF ACTION OF LACTOCOCCIN PRODUCED BY *LACTOCOCCUS LACTIS R*

LACTOCOCCUS LACTIS R TARAFINDAN ÜRETİLEN LACTOCOCCIN R'IN ETKİSİ MEKANİZMASI

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ABSTRACT: It was found that lactococcin R adsorbed to all gram-positive but not to the gram-negative bacteria tested and its adsorption was dependent on pH. It was observed that the binding of lactococcin R was prevented by anions of several salts (Cl^-, $PO^{-3}4$) and lipoteichoic acid. It was determined that pretreatments of sensitive cells and cell walls with detergents, organic solvents or enzymes did not reduce subsequent binding of lactococcin R. However, treatment of cell wall preparations with methanol:chloroform and hot 20% TCA caused such walls to lose their ability to adsorb lactococcin R. It was found sensitive cells treated with lactococcin R lost high amounts of intracellular K^+ ions, UV-absorbing materials and became more permeable to ONPG. In addition, it was observed that different lactococcin R concentrations (0-2560 AU/ml) decreased the colony counts of Listeria monocytogenes by 99% and also caused a reduction in the absorbance values of L. monocytogenes. This results show that the mode of action of lactococcin R is bactericidal rather than bacteriostatic.

ÖZET: Lactococcin R test edilen tüm gram-pozitif bakterilere adsorbe olmasına karşın gram-negatif bakterilere adsorbe olmadığı ve adsorpsiyonun pH'ya bağlı olduğu bulunmuştur. Lactococcin R'ın adsorpsiyonu, bazı tuzların anlyonları (Cl^-, $PO^{-3}4$) ve lipoteichoic asit tarafından önlendiği tespit edilmiştir. Hücrelerin ve hücre duvarlarının deterjan, organik solventler veya enzimlerle ön işleme tabi tutulması lactococcin R'ın bunlara bağlanmasını azaltmadığı gözlenmiştir. Fakat, metanol-kloroform ve sıcak %20 TCA ile muamele edilen hücre duvarı preparatları lactococcin R'ı bağlama yeteneklerini kaybettiği tespit edilmiştir. Lactococcin R ile muamele edilmiş duyarlı hücreler çok fazla miktarda intrasellüler K^+ lyonu ve UV-absorbe edilebilen materyalleri kaybettiği, ve ONPG'ye karşı daha çok geçirgen olduğu bulunmuştur. Farklı lactococcin R konsantrasyonları (0-2560 AU/ml), Listeral monocytegenes koloni sayısını yaklaşık %99 oranında azaltığı ve ayrıca L. monocytogenes'in absorbans değerinde de azalmaya neden olduğu tespit edilmiştir. Bu sonuçlar lactococcin R'ın etki mekanizmasının bakteriyostatikten daha ziyade bacteriyosidal olduğunu göstermektedir.

INTRODUCTION

Bacteriocins from lactic acid bacteria are ribosomally synthesized cationic and amphiphilic antimicrobial peptides. Their antimicrobial activity is directed towards sensitive gram-positive bacteria, some of which are associated with food spoilage and foodborne illness, such as Staphylococcus aureus and Listeria monocytogenes (TAGG et al. 1976, KLAENHAMMER 1993). Their production has been described in bacteria belonging to all main genera of the lactic acid bacteria group, lactococci, lactobacilli, leuconostoc, pediococci and carnobacterium (BAREFOOT and KLAENHAMMER 1984, BHUNIA et al. 1988, PIARD et al. 1992, AYMERIC et al. 1996, VUYST et al. 1996, CASAUS et al. 1997). The interest in bacteriocins increased considerably because they might serve as food preservatives able to replace current chemical agents from which potentially carcinogenic compounds can arise.

YILDIRIM and JOHNSON (1998) have described a bacteriocin, termed lactococcin R from Lactococcus lactis R. lactococcin R inhibits the growth of selected species of the genera Listeria, Staphylococcus, Clostridia, Bacillus, Micrococcus, Enterococcus, Lactobacillus, Leuconostoc and Pediococcus. It is sensitive to some proteolytic enzymes but resistant to pH (2-10), heat and organic solvents. Lactococcin R is produced maximally during late stationary and early log growth phase.

The objectives of this study were to determine mode of action of lactococcin R and factors affecting its mode of action and its adsorption to indicator organisms.

2. MATERIALS AND METHODS

2.1. Bacterial culture and preparation of lactoccocin R. *Lactococcus lactis* R was cultured and stored in de Mann Rogosa Sharpe (MRS) broth with 20% glycerol at -70°C. To prepare lactococcin R, *L. lactis* R was inoculated into MRS broth (1.5 L) at the rate of 1 % (v/v). After 22 h incubation at 30°C, cell free-supernatant collected by centrifugation (1,000 x g for 20 min) was passed through sterile filters (0.45 µm pore size), freeze dried and reconstituted to 150 ml with sterile distilled water. These preparats were partially purified using Micro-Cel (COVENTRY et al. 1996, YILDIRIM et al. 1999).

2.2. Cell wall preparation. Cell walls of L. plantarum NCDO 955 cells grown in 200 ml of MRS broth at 30°C for 18-20 h were prepared using the method described in else where (SPROTT et al. 1996, YILDIRIM et al. 1999).

2.3. Assay of adsorption of lactococcin R to indicator organisms. Adsorption of lactococcin R to indicator cells was studied by procedure described previously (BHUNIA et al. 1991, YILDIRIM et al. 1999).

2.4. Effect of temperature, pH, time, NaCl, sodium phosphate, lipotelchoic acid and various chemicals on adsorption of lactococcin R to *L. plantarum*. The effect of temperature (0, 10, 25, 50 or 85°C), pH (2, 3, 4, 5, 6, 7, 8, or 9), time (0.5, 5, 10, 20, 30 or 60 min), NaCl and sodium fosfat NaCl (0, 25, 50, 100, 200, 300, 400, or 500 mM) on adsorption of lactococcin R were determined using the method of BHUNIA et al. (1991) and YILDIRIM et al. (1999).

L. plantarum cells were suspended in purified lipotelchoic acid from Enterococcus faecalis (Sigma) (0.1 ml of 10 mg/ml in distilled water) and different salt or protein solutions, and then mixed with lactococcin R (5,120 AU). After incubation at 25°C for 30 min, cells were centrifuged at 7,000 g for 15 min. The supernatants were assayed for remaining lactococcin R activity to calculate the percentage of adsorption (BHUNIA et al. 1991, YILDIRIM et al. 1999).

2.5. Effect of some detergents, solvents and enzymes on adsorption of lactococcin R to whole cell and cell walls of *L. plantarum*. The effects of detergents, solvents and enzymes on adsorption of lactococcin R to whole cells and cell walls were determined as described previously (BHUNIA et al. 1991).

2.6. Effect of lactococcin R on the growth of *L. monocytogenes*. For examining the effect of lactococcin R on actively growing *L. monocytogenes* cells in BHI broth, various concentrations of lactococcin R (0, 640, 1,280 or 2,560 AU/ml) were added to each sample. The samples were incubated at 37°C for 4 h. Samples were periodically withdrawn to determine the cell viability and sample absorbance at 600 nm.

2.7. Effect of lactococcin R treatment on the release of cytoplasmic materials in the environment. Pure lactococcin R (640 AU/ml) was added into *L. plantarum* cell suspension in 5 ml of phosphate buffer (5 mM, pH 5.5). The controls were cells without bacteriocins and buffer with lactococcin R but no cells. After incubation at 37°C for 45 min, the samples were centrifuged, filtered through 0.22 µm pore size sterile membranes (Millipore) and read at 260 nm in a Beckman DU-50 spectrophotometer. The concentration of potassium ions in the filtrates was measured by atomic adsorption spectroscopy.

2.8. Measurement of o-nitrophenol-ß-D-galactopyranoside (ONPG) permeability on *L. plantarum* cells treated with lactococcin R. *Pure lactococcin* R (640 AU/ml) was added to *L. plantarum* cells (2ml) in phosphate buffer (5mM, pH 5.5). After incubation at 25°C for 5 min, each sample received 0.1 ml of ONPG (0.1 M) and was incubated at 37°C for 10 min. The absorbances of the cell free supernatant fluids collected by centrifugation (7,000 g for 15 min) were measured at 420 nm in a Beckman DU-50 spectrophotometer. Controls were either *L. plantarum* cells or lactococcin R in the same buffer. ONPG concentration was determined using the following equation (BHOWMIK et al. 1987): ONP (mM)= A_{420nm}x 455.

3. RESULTS

3.1. Adsorption of lactococcin R to indicator organisms. Lactococcin R was adsorbed on cells of all gram-positive bacteria tested, both sensitive and resistant, and adsorption ranged from 75-100 % (Table 1). However, lactococcin R did not adsorb to the gram-negative bacteria tested.

Table 1. Adsorption of Lactococcin R to Resistant and Sensitive Bacterial Species.

Sensitive organisms	Adsorption (%)	Resistant organisms	Adsorption (%)
Lactobacillus plantarum	100	Lactococcus lactis	94
Pediococcus dextrinicus	100	Pediococcus cerevisiae	100
Leuconostoc oenos	100	Staphylococcus aureus	90
Enterococcus faecalis	100	Salmonella typhimurium	0
Listeria monocytogenes	95	Escherichia coli	0
Listeria ivanovii	95	Yersinia enterocolitica	0
Bacillus cereus	100	Pseudomonas fluorescens	0

3.2. Effect of pH, temperature, time, lipoteichoic acid and various chemicals on adsorption of lactococcin R. The adsorption of lactococcin *R to L. plantarum* cells was pH dependent. It was found that the adsorption of lactococcin R was maximum (100 %) between pH 5.0 and 7.0. However, the adsorption was reduced to about 50 to 25% at pH values below or above.

The amount of binding of lactococcin R to L. plantarum under different temperature conditions (0, 10, 50 or 85°C) were found to be the same as for the control (100%) maintained at 25°C. High (85°C) and low temperature (0°C) treatments of cells had no effect on lactococcin R adsorption.

After exposure to crude lactococcin R for 0.5, 5, 10, 20, 30 or 60 min, the growth rate of *L. plantarum* was zero for the duration of the experiment time (4 h) (Fig. 1). The control cells under the same condition grew very well. Ninety percentage of lactococcin R was adsorbed in 0.5, 5, or 10 min, and adsorption reached 100% after 20 min.

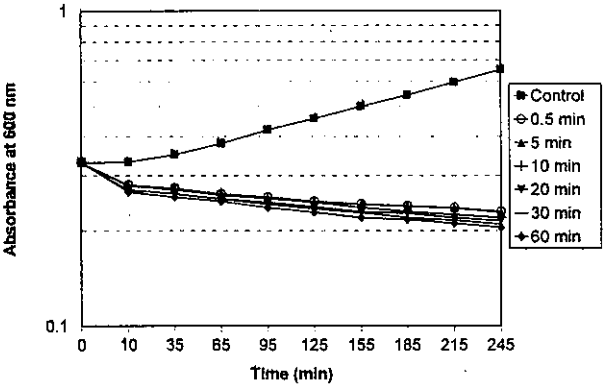

Fig. 1. Effect of lactococcin R (5120 AU/ml) treatment for times varying from 0.5 to 60 min on the growth of *L. plantarum*

Table 2. Effect of the Presence of Lipoteichoic Acid and Various Chemicals on Adsorption of Lactococcin R to L. Plantarum.

Treatments	Adsorption (%)
Control (cells+lactococcin R)	100
Lipoteichoic acid (1 mg/0.1 ml)	0
NH_4-citrate (0.1 %)	100
NH_4Cl (0.3 M, pH 5.5)	85
$MgCl_2$ (0.3 M, pH 6.5)	75
KCl (0.3 M, pH 5.6)	85
KI (0.3 M, pH 6.6)	85
Na-acetate (0.5 %)	100
$NaCO_3$ (0.3 M, pH 7.6)	90
EDTA (0.01 M)	100
Tris-HCl (0.6 M, pH 6.8)	50
Tris-HCl (0.02 M, pH 6.8)	85
Bovine serum albumin (0.5 %)	100
Casein (0.5 %)	100

* At pH 7 and 8 adsorption of lactococcin R were about 100 and 75 % respectively.

The presence of purified lipoteichoic acid (from E. faecalis) completely inhibited the adsorption of lactococcin R to L. plantarum cells (Table 2). Presence of NH_4Cl, $MgCl_2$, KCl, KI or Tris-HCl reduced lactococcin R adsorption to *L. plantarum* cells, while NH_4-citrate, Na-acetate, $NaCO_3$, EDTA, bovine serum albumin or casein did not reduce adsorption (Table 2).

3.3. Effect of NaCl and sodium phosphate on adsorption of lactococcin R. The presence of NaCl at 25 to 50 mM had no effect on both adsorption and lethal effect of lactococcin R (Fig. 2A). However, higher concentrations (100 mM or above) adversely affected the adsorption of lactococcin R and lead to higher viable cell counts. Similarly, phosphate concentrations above 100 mM reduced the adsorption and lethal effect of lactococcin R (Fig. 2B). Lactococcin R (1,280 AU/ml) caused 99.97 %

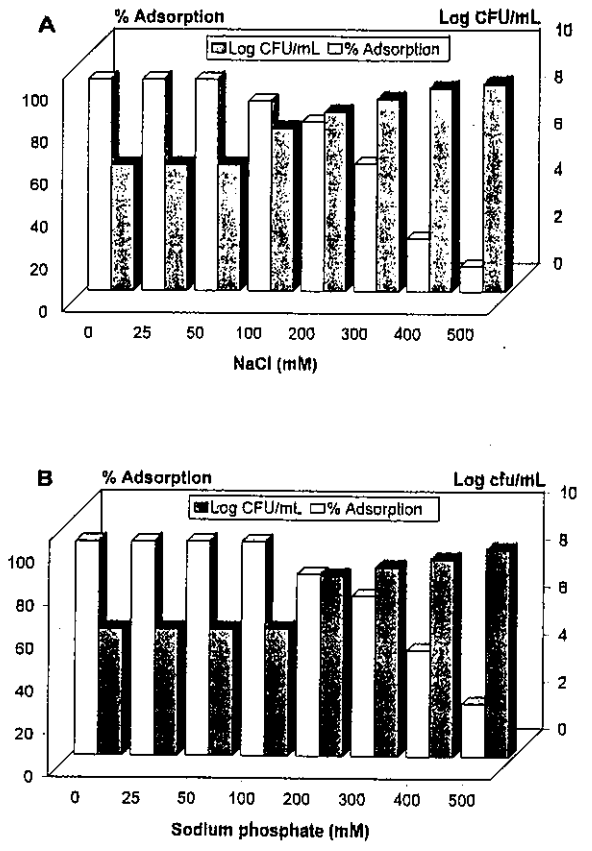

Figure 2. Effect of NaCl and Na-phosphate on adsportion of lactococin R and on viability of *L. plantarum*. A, NaCl; B, Na-phosphate. Cell count was 5,5x10^8 and concentration of bifidocin B added was 1280 AU/ml.

death of control cells. Addition of 500 mM of NaCl or sodium phosphate reduced the activity of lactococcin R and reduced target cell death to 20.6 or 41.1%, respectively. These protective effects of NaCl and phosphate could be overcome by increasing the concentration of lactococcin R to 5,120 AU/ml (Table 3).

3.4. Effect of some detergents, solvents and enzymes on adsorption of lactococcin R. Treatment of whole cells and cell walls with guanidine-HCl (4M), SDS (1%), triton-X (2%), organic solvents such as mercaptoethanol, ethanol, hexane, acetone and methanol, and enzymes (protease, trypsin, lipase, lysozyme) had no effect on adsorption of lactococcin R. However, treatment of cell walls with a mixture of methanol and chloroform followed by hot 20% TCA prevented the adsorption of lactococcin R. The later treatment of methanol: chloroform and with cold 20% TCA treatment only reduced adsorption by 20%, whereas these treatments on whole cells had no effect on lactococcin R adsorption.

3.5. Effect of lactococcin R on the growth of *L. monocytogenes*. Addition of lactococcin R at various concentrations (0-2560 AU/ml) to a growing culture of L. monocytogenes in BHI broth resulted in reductions of the colony counts and absorbance values (600 nm) in proportion to the amount of lactococcin R added (Fig. 3). After 30 min incubation period, the counts had decreased approximately by 1.2, 2.2 and 3.1 log at concentration of 640, 1280 and 2560 AU/ml, respectively (Fig 3). After 4 h, the colony forming units declined 999% at all lactococcin R concentrations.

Table 3. Effect of Different Lactococcin R Concentration on Viability of Lactobacillus Plantarum in the Presence of NaCl or NaH_2PO_4

Lactococcin R	500 mM NaCl		500 mM NaH2PO$_4$	
(AU/ml)	CFU/ml	Cell death (%)	CFU/ml	Cell death (%)
0	6.5x10^8	0.00	6.5x10^8	0.00
1280	5.1x10^8	21.54	3.8x10^8	41.54
2560	2.4x10^8	63.08	1.4x10^8	78.46
5120	9.8x10^6	98.49	6.3x10^6	99.03
10240	1.1x10^6	99.80	4.4x10^5	99.93

3.6. Effect of lactococcin R on the release of cellular materials. The treatment of *L. plantarum* with pure lactococcin R caused the leakage of UV-absorbance materials and potassium ions, and an increase in 260 nm absorbing materials in contrast to control samples (Table 4). Potassium ion efflux and UV-absorbing materials increased 13 and 14 fold in lactococcin R treated cells, respectively. In addition, the cells treated with lactococcin R became eight times more permeable to ONPG and ONP than the untreated cells (Table 4).

4. DISCUSSION

In this study the possible mode of action of lactococcin R was determined. Lactococcin R was adsorbed to all sensitive or resistant gram-positive bacteria, but not to any of the gram-negative bacteria tested. Gram-negative bacteria probably do not have the necessary adsorption binding sites (receptors). The adsorption of lactococcin R to the gram-positive resistant cells could be due to the presence of non-specific (non lethal) receptors on such strains (UPRETTI and HINSDILL 1975, WICKEN and KNOX 1975). The sensitive cells probably have both non-specific and specific (lethal) receptors. Adsorption of lactococcin R caused cell death in sensitive gram-positive cells, but did not cause death when adsorbed to the resistant cells. The adsorption and lethal action of lactococcin R on sensitive gram-positive strains was very rapid. In 0.5 min, it was adsorbed to L. plantarum cells by 90%. In addition, adsorption of lactococcin R was dependent on pH rather than time.

Table 4. Effect of Lactococcin R on Release of Cellular Materials From *L. Plantarum* Cells

Parameters	Untreated cell supernatant	Lactococcin R solution	Treated cells supernatant fluid
Absorbance (260 nm)	0.152	0.141	2.14
Potassium ions (mg/l)	36	2.84	468
ONP (mM)	193	0	1569

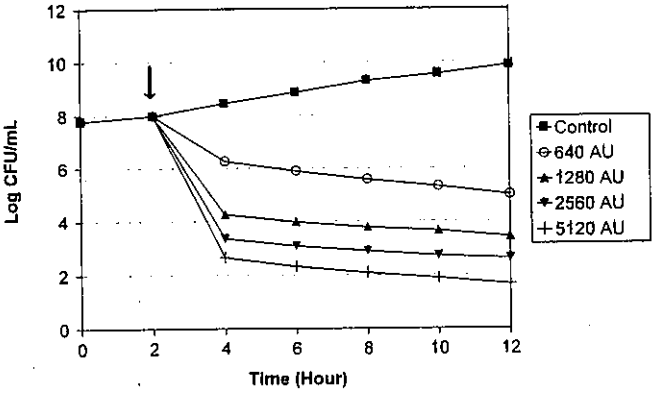

Figure 3. Effect of lactococcin R on growing cells of *L. plantarum*. Lactococcin R was added at 2 h (arrow).

The presence of phosphate and chloride ions caused a reduction in adsorption of lactococcin R to the cell surface and the reduction was concentration dependent. This protective effect of chloride and phosphate was

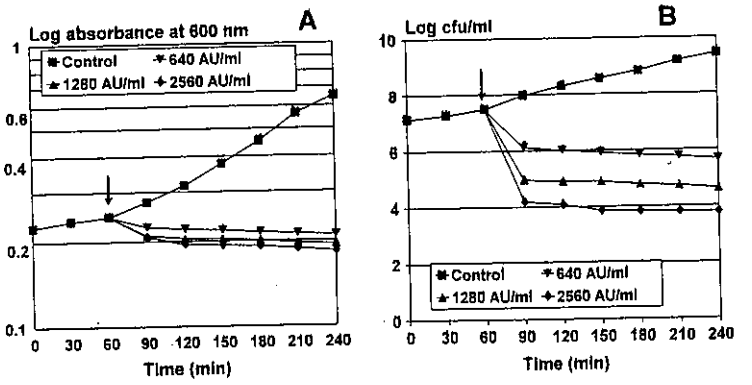

Figure 3A-B.: Effect of lactococcin R on the growth of *L. monocytogenes* in BHI broth at 37°C. Bacteriocin was added at 60 min (arrow). Panel A, log absorbance at 600 nm; panel B, log CFU/ml.

prevented by increasing the concentration of lactococcin R. These inhibition patterns show that ionic interactions may involve the binding of lactococcin R to cell surface receptors. Cells or cell wall preparations of *L. plantarum* treated with different enzymes, several organic solvents or detergents did not lose ability to adsorb lactococcin R. These results demonstrate that the major binding sites of lactococcin R might not be proteins, carbohydrates or lipids. However, cell wall preparations treated with methanol:chloroform and hot 20% TCA lost the ability to adsorb lactococcin R. This indicates the possible involvement of cell wall lipoteichoic acid (LTA) in binding of lactococcin R (BHUNIA et al. 1991, PUCCI et al. 1988). Also, the addition of purified

LTA to cells blocked the adsorption of lactococcin R. As for the results for other bacteriocins (BHUNIA et al. 1991, YILDIRIM et al. 1999,), lactococcin R probably forms a complex with LTA making unavailable for adsorption to the target cells and thus unable to cause cell growth inhibition or death. This also explains why lactococcin R was adsorbed to gram-positive bacteria and not to gram-negative bacteria. LTA molecules probably are the non-specific receptors and are associated with binding of lactococcin R in both resistant and sensitive gram-positive bacteria. In the sensitive cells, when these sites have been saturated, lactococcin R binds to specific receptor(s) and caused cellular changes associated with cell death (BHUNIA et al. 1991, YILDIRIM et al. 1999). The lethal action of lactococcin R on sensitive cells results in the loss of some cellular materials, UV-absorbing materials and potassium, associated with membrane damage. Similar results reported by other researchers (PUCCI et al. 1988, BHUNIA et al. 1991, YILDIRIM et al. 1999).

Lactococcin R is bactericidal to sensitive bacterial cells and this effect is produced within a few minutes. Cell death appears to be associated with lysis of the cell membrane or subsequent cytosol leakage. In a word, the mode of action of lactococcin R first involves the binding of lactococcin R to some non-specific receptors like lipoteichoic acid and then, followed by binding to specific receptors which can cause changes in the membrane integrity. As a result, the cells lose potassium ions, UV-absorbing materials and other small molecules. The cell membrane also loses structural integrity and lyses.

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