PLASMID-ASSOCIATED LACTOCIN RN78 PRODUCTION IN A LACTOBACILLUS RN78 STRAIN ISOLATED FROM A DAIRY SAMPLE IN IRAN

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SUMMARY: A bacteriocin, lactocin RN78 produced by a Lactobacillus RN78 strain isolated previously from a cheese sample, was found inhibitory against many pathogenic Gram-negative and Gram-positive bacteria including Kl. pneumoniae, L. ivanovii, L. monocytogenes, Ps. aeruginosa, and S. aureus. Characterization of the inhibitory agent produced by RN78 strain revealed it to be heat stable (100 and 121°C for 90 and 15 minutes respectively), and active at wide pH range of 3 to 9. Moreover, the inhibitory activity was completely destroyed after treatment with proteolytic enzymes (Pronase and Trypsin); while no change in its antimicrobial activity was recorded after treatment with the enzyme catalase. A rapid cell death of the indicator strain (10^6 to 10^7 cells) within 2 hours of incubation in presence of 10240 AU mL^{-1} of lactocin RN78 indicated the bactericidal nature of this bacteriocin against L. monocytogenes. Treatment of lactocin RN78 with 0.5 mM EDTA resulted in expansion of the inhibitory spectrum and it inhibited the growth of E. coli and S. typhi to which it was previously non-influential. Curing of the producer strain RN78 with acridine orange resulted in Bac-mutant defective in bacteriocin production. Plasmid analysis of the wild type and mutant's defective in bacteriocin production indicated the absence of a 42Kb plasmid that was originally present in the wild producer strain. All the cured strains retained their immunity to lactocin RN78 which indicated the possibility of the immunity genes to be on the chromosomes instead of being on the plasmid RN.

Key Words: Lactobacillus, Inhibitory agent, Lactocin, Bacteriocin, Curing, Plasmids.

INTRODUCTION

Lactic acid bacteria (LAB) produce biologically active peptides or protein complexes that display a bactericidal mode of action almost exclusively toward Gram-positive bacteria and particularly toward closely related species (1-3). These antibacterial substances of a proteinaceous nature that are produced by different bacterial species of LAB are defined as bacteriocins, and are found to be active against food spoilage and food borne pathogenic microorganisms including *B. cereus*, *Cl. perfringens*, *S. aureus*, and *L. monocytogenes* (4-6). Among bacteriocins so far characterized, nisin is the best defined, and the only purified bacteriocin produced by *Lactococcus lactis*, that has been approved for use in food products in almost 45 countries (7, 8). Besides nisin, pediocin has also been approved for use in food products. The main properties of

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nisin and pediocins are there broad inhibitory spectrum with high antimicrobial activity and high stability at low pH and high temperatures (9, 10). Moreover, these antibacterial compounds were found stable over several months during frozen and refrigeration storage and after drying (10). Several other bacteriocins from lactic acid bacteria, such as lactococcin from *Lactococcus lactis* subsp. lactis 484 (11), acidocin 8912 from *Lactobacillus acidophilus* TK8912 (12,13), plantaricin A from *Lactobacillus plantarum* (14), gassericin A from *Lactobacillus gasseri* LA39 (15) and plantaricin-149 from *Lactobacillus plantarum* NRIC 149 (16) have been detected, purified and characterized.

The determination of optimum parameters for both enhanced production and purification of bacteriocins is among the prerequisites for their use in the food, veterinary and pharmaceutical industries. In the present study, we investigated the effect of variable parameters on a wide spectrum plasmid linked bacteriocin, lactocin RN78 produced by an indigenous strain of lactobacilli isolated from a milk sample for its future exploitation as a food preservative.

MATERIALS AND METHODS

Bacterial strain, media and culture conditions

Lactobacillus strain isolated from a cheese sample in a previous study was used as bacteriocin producing strain. This strain was grown in MRS broth (Merck, Germany) at 37°C for 24 hours, while other gram positive and negative bacteria used were grown in nutrient broth (Merck, Germany) and Brain heart infusion (BHI, Himedia, India) at 37°C.

Bacteriocin bioassay and spectrum of inhibition

Bacteriocin screening was performed by using the agar well diffusion method described by Schillinger and Lüke (17). To quantify the minimum inhibitory concentration of Lactocin RN78 against a number of gram positive and gram negative sensitive cells, the critical dilution assay described by Mayr-Hartings *et al.* (18) was used. The soft agar lawn containing 10^6 to 10^7 of indicator culture were overlaid on solidified agar plates and serial two-fold dilution of supernatant fluid made in fresh MRS broth was poured in individual wells punched in these agar plates. All the plates were observed for zone of inhibition around the wells after 18-24 hours of incubation at 37°C. One AU (arbitrary unit) was defined as reciprocal of the highest serial two fold dilution showing inhibition of the indicator strain and was indicated in AU mL^{-1}.

Effect of enzymes, pH, detergents and heat treatment

To characterize lactocin RN78, the effect of variable factors were studied on the inhibitory agent. The producer strain was grown in MRS broth at 37°C for 24 hours, the cells harvested by centrifugation (10,000 rpm, 10 minutes 4°C), and the cell-free supernatant adjusted to pH 6.0. Samples of 1 mL were incubated for 2 hours in the presence of mg/mL^{-1} (final concentration) trypsin, Pronase E and Catalase (Boehringen Mannheim GmbH), respectively, and tested for antimicrobial activity.

The effect of pH on the bacteriocin was tested by adjusting the cell-free supernatants from pH 3.0 to 12.0 with sterile 1M HClA or 4M NaOH. After 24 hours of incubation at 37°C, all samples were tested for antimicrobial activity.

Thermostability of lactocin RN78 was evaluated by heating the supernatant fluid at 80, 100 and 121°C, respectively. Residual bacteriocin activity was tested after every 10 minutes.

In a separate experiment the effect of surfactants on the bacteriocin was tested by adding Sodium dodecyl sulphate (SDS), and Tween 20, Tween 80 (1% v/v, final concentration), respectively, to the cell-free supernatant. EDTA was added to the cell-free supernatant to yield a final concentration of 0.5 mM and 2.0 mM, respectively. Untreated cell-free supernatant and the detergents at these respective concentrations were used as controls. All samples were incubated at 37°C for 4 to 6 hours and then tested for antimicrobial activity by agar well diffusion method.

Survival assay

A two fold dilution of neutralized culture supernatants of RN78 was prepared in MRS broth and incubated at 37°C after addition of 50 μl of overnight culture of indicator cells (10^6 to 10^7 cells). At every 30 minutes of time interval the absorbance (O.D) at 660 nm, the viable count (cfu mL^{-1}), and the antibacterial activity (AU mL^{-1}) assayed.

Stability of bacteriocin during storage

Lactocin RN78 was analyzed for its stability at different temperatures during long term storage. The bacteriocin was incubated in small vials at -20, 4, and 37°C respectively and the antimicrobial activity determined every month by agar well diffusion method.

Plasmid curing and detection

Lactobacillus RN78 mutant's defective in bacteriocin production were isolated by curing experiments as described by Hirota (19). Acridine orange was used as chemical curing agent at a final concentration of 5-150 μg mL^{-1}. All the colonies after treatment were picked carefully and checked for inhibitory activity by agar well diffusion method. The colonies showing no zone of inhibition were selected as Bac-mutants and screened further for the presence of plasmids.

The presence or absence of plasmid in the wild and Bac-mutants of lactobacillus RN78 was analyzed by Echardt gel electrophoresis (20) with slight modifications. The cells of the test strain (100-500 μL) were collected by centrifugation and washed

Table 1: The inhibitory spectrum of lactocin RN78 against a number of gram positive and gram negative bacteria.

No	Indicator organisms	Gram reaction	Inhibitory activity
1	*B. subtilis*	G+	-
2	*L. casei*	G+	+
3	*L. bulgaricus*	G+	+
4	*L. ivanovii*	G+	+
5	*L. monocytogenes*	G+	+
6	*S. typhi*	G+	-
7	*S. aureus*	G+	+
8	*S. agalactiae*	G+	-
9	*S. pyogenes*	G+	-
10	*S. dysagalactiae*	G+	-
11	*E. coli*	G-	-
12	*Kl. pneumoniae*	G-	+
13	*P. multicida*	G-	-
14	*Ps. aeruginosa*	G-	+
15	*S. dysentriae*	G-	-

+: Zone of inhibition
- : No zone of inhibition

with ice cold TNE buffer (10 mM Tris, 100 mM NaCl and 1 mM EDTA). All were suspended in 100 μL TNE buffer (10 mM Tris and 1 mM EDTA) and added to the slots made in 0.9% agarose gel. 50 μL of lysis solution (25% w/v sucrose solution in TBE, 10 mg/mL^{-1} lysozyme and 1 unit RNase) were added to the suspended cells in the slots and mixed carefully with a sterile toothpick. The gel was run for 30 min at 30V and then at 120V for approximately 3 hours and observed under UV.

RESULTS AND DISCUSSION

In the last decade, there has been extensive research in use of lactic acid bacteria and their metabolites to control pathogenic and perishing microorganisms in foodstuffs. The bacteriocins produced by LAB may be promising for use as bio-preservatives due to their inhibitory spectrum against many important food borne pathogens (21-23). In this research we were able to isolate and characterize a broad spectrum bacteriocin named lactocin RN78, produced by lactobacilli RN78. Table 1 indicates the inhibitory spectrum of the cell-free supernatant fluid of RN78 against variety of gram positive and gram negative pathogens. The antibacterial substance produced by RN78 strain was inhibitory towards

Figure 1: The antibacterial activity demonstrated by Lactocin RN78 against *L. monocytogenes* in agar-well diffusion assay.

one of the important food borne pathogens such as *L.monocytogenes* (Figure 1). Bacteriocin M46, nisin, mesentericin Y105, curvacin A, carnosin, enterocin 1146, sakacin P, piscicolin 126 *etc.* are some of the bacteriocins produced by lactic acid bacteria that have been shown to be inhibitory towards *L. monocytogenes* (22-24).

Most of the LAB bacteriocins have been reported to be inactive against gram negative bacteria (3). However, contrasting to these reports, lactocin RN78 appeared inhibitory towards some of the gram negative bacteria tested in this study like *Kl. pneumoniaea* and *Ps. aeruginosa*. Moreover, this spectrum of inhibition against gram negative bacteria was enhanced by treatment with some of the detergents like EDTA, Triton X100, Tween 20, Tween 80, and SDS *etc.* (9,15). The inhibitory activity of the bacteriocin RN78 after treatment with 0.5 mM EDTA, was expanded against *E. coli* and *Salmonella typhi* to which they were previously unaffected. This enhanced activity of lactocin RN78 after treatment with detergents could be attributed to the dispersion of bacteriocin molecule into small active subunits, which ultimately resulted in more lethal hits and increased inhibition. In accordance with our findings Muriana and Klaenhammer (6) and Kato *et al.* (25) had also reported that the bacteriocins become more active when treated with SDS or other detergents, as it disperses the protein molecule large units into small active subunits resulting in more lethal hits. However, contrasting results have also been reported by Tagg and his co-workers (26), who concluded that the bacteriocins

Table 2: The effect of physical and chemical factors on the activity of lactocin RN78.

No	Agent	Inhibitory activity
1	**Enzymes**	
	Pronase	-
	Trypsin	-
	Catalase	+
2	**Detergents**	
	SDS	+
	EDTA	+
	Tween 80	-
3	**Temperature**	
	80°C	+
	100°C	+
	120°C	+
4	**pH**	
	3.0	-
	5.0	+
	7.0	+
	10.0	+

present in large subunits when dispersed into smaller subunits by detergents instead of enhancing their activity might result in reduction or complete inhibition of their antibacterial activity.

The antagonistic substance produced by RN78 was completely inactivated by proteolytic enzymes such as Trypsin and Pronase (Table 2). These results reflect the proteinaceous nature of lactocin RN78. In contrast, catalase had no effect on the antagonistic agent, which rules out the possibility that inhibitory activity of lactocin RN78 is due to hydrogen peroxide.

Many of these bacteriocins widely studied have been reported to be active in a wide pH range and are temperature tolerant (10, 17). A novel property of lactocin RN78 was its ability to resist 100°C for more than an hour (90 minutes) and autoclaving temperature of 121°C for 15 minutes. Many heat resistant *Lactobacillus* bacteriocins reported previously are LP27 (24), lactacin B (1) and enterocin 101 (24), which were stable at 100°C for 60 minutes. Lactocin RN78 seems to be a more resistant bacteriocin than the above two. The heat stability of this bacteriocin might be attributed to the complex nature of lactocin RN78 protein molecule. The pH profile of lactocin RN78 indicated the inhibitory protein to be pH tolerant, as it retained its activity at pH values ranging from 3-10, with maximum activity between pH 5.0 and 7.0.

Figure 2 illustrates the survival of the indicator cells in the presence of the lactocin RN78. The bacteriocin in study was shown to cause rapid cell death when added to actively growing cells of sensitive organisms. The bactericidal mode of action of lactocin RN78 was examined using 10^6 to 10^7 of the actively growing cells of *L. monocytogenes* as indicator cells and adding 10240 AU mL^{-1} of the said bacteriocin. The results indicated a rapid loss of viability of sensitive cells in comparison to control cultures to which no bacteriocin was added. Almost 99.9% of the sensitive cells were killed within 2 hours of incubation, demonstrating the potency of this bacteriocin against this pathogen. Moreover, by adding increasing concentrations of bacteriocin, and increased rate of this killing effect was observed (e.g., only 1 of 10^6 of the sensitive cells survived exposure to 12,840 AU mL^{-1} of lactocin RN78, within an hour of incubation), indicating that the residual populations are not resistant and can be killed by merely increasing the bacteriocin concentration. Similar observations were made by other researchers who reported a decrease in viability of the sensitive cells with increasing concentrations of bacteriocin (10).

Figure 2: The survival of *L. monocytogenes* in the presence of lactocin RN78.

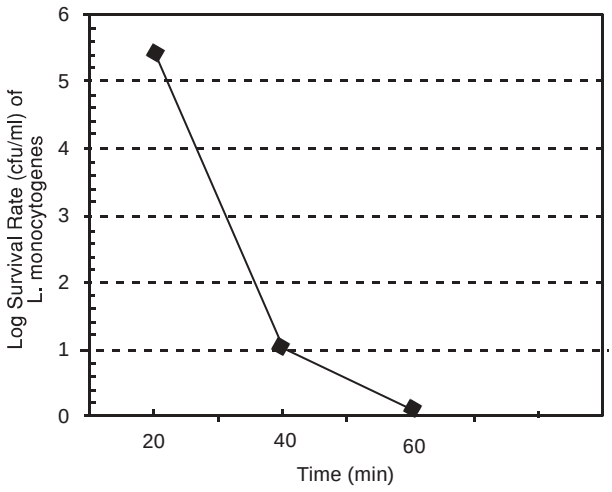
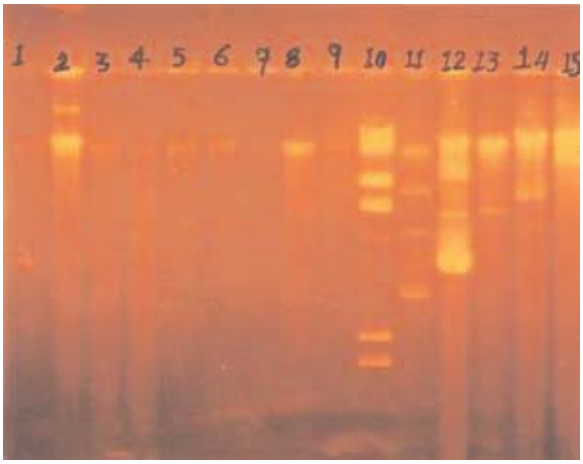


Figure 3: The plasmid analysis of wild type and cured colonies of Lactobacillus RN78 strain.



Lactocin RN78 seemed to be stable at -20°C for more than two years, when incubated at the said temperature in skim milk with 5% glycerol. The stability of this bacteriocin was also observed at 4 and 37°C for a year and a month time, respectively.

Bacteriocin structural genes have been shown to be either plasmid or chromosome-borne (27, 28). Preliminary results indicate that lactocin RN78 production in RN78 strain might be plasmid linked as the loss of plasmid in Bac-mutants was verified by employing Echardt gel electrophoresis. More of the mutants seemed to posses the plasmid, although the presence of a 42Kb plasmid was verified in the wild producer strain (Figure 3). These results gave an early indication of plasmid linked lactocin RN78 production in the test strain. When the immunity of the bacteriocin was checked to the cured strain it appeared to retain its resistance as were still not inhibited by the bacteriocin. All these results suggest that the immunity genes for lactocin RN78 might be on the chromosomes and not on the isolated plasmid.

CONCLUSIONS

To conclude we might suggest that since the genetic determinants for lactocin RN78 are encoded on a large self-transmissible plasmid, the bacteriocin genes may be conveniently transferred to different lactococcal starters. The resulting food-grade strains can then be used to make a significant impact on the safety and quality of a variety of fermented foods, through the inhibition of undesirable microflora. The bacteriocin is heat stable so it can also be used as an ingredient in a powdered from such as a spray-dried fermentate. Given the observation that lactocin RN78 is effective at physiological pHs, there is also considerable potential for biomedical applications. Strains which produce these inhibitors can be exploited in the acceleration of cheese ripening by assisting the premature lysis of starter cultures. Generally they are active against food spoilage and foodborne pathogenic microorganisms including *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, and *Listeria monocytogenes* and thus there is an increased tendency to use naturally occurring metabolites to prevent the growth of undesirable flora in foodstuffs. These natural metabolites could replace the use of chemical additives such as sorbic acid, sulfur dioxide, nitrite and others and be used as bio-preservatives. Moreover, since the spectrum of activity of lactocin RN78 was enhanced by combination with chelating agents, there is considerable interest in using such bacteriocins in current and potential applications in the veterinary and pharmaceutical area.

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