DETECTION AND CHARACTERIZATION OF A HEAT STABLE BACTERIOCIN (LACTOCIN LC-09) PRODUCED BY A CLINICAL ISOLATE OF LACTOBACILLI

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SUMMARY: A new bacteriocin lactocin LC-09 produced by Lactobacillus strain LC-09, isolated from a clinical sample, was inhibitory against many species of lactobacilli and other Gram-positive bacteria including Listeria ivanovii, Streptococcus agalactii and S. peyogenes. Characterization of the bacteriocin revealed it to be extremely heat stable (4 hours at 100°C) and active at acidic pH values. This bacteriocin was inactivated by protease treatment. Curing of LC-09 with acridine orange, ethidium bromide and by elevated temperature (40°C) resulted in mutants defective in bacteriocin production.

Key Words: Heat stable, inhibitory, protease.

INTRODUCTION

Lactic acid bacteria (LAB) are used extensively in food processing such as dairy, beverage and meat products (16). One of the most important roles of lactic acid bacteria is to inhibit growth of spoilage and pathogenic bacteria in food (9). Lactic acid bacteria can produce a variety of antibacterial agents, including organic acids, diacetyl and hydrogen peroxide (10,13,14). Some lactic acid bacteria are well known to produce bacteriocins (7,11,23). Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to producer bacterium (21). Certain bacteriocins produced by lactic acid bacteria prevent growth of a variety of food-borne pathogens including *Listeria monocytogenes* (2,3,19).

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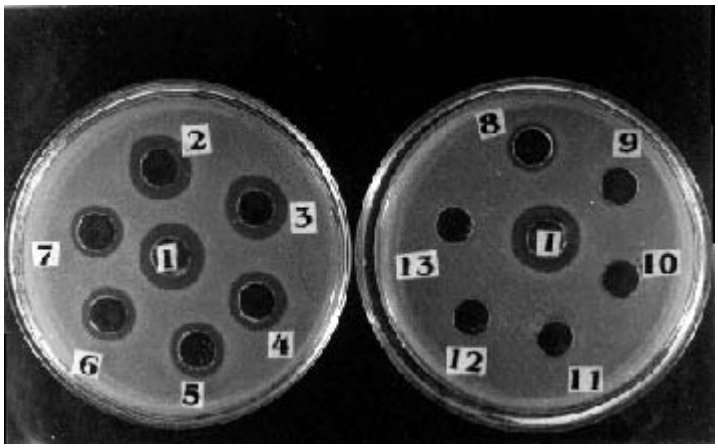
Several bacteriocins from lactic acid bacteria, such as lactococcin from *Lactococcus lactis* subsp. lactis 484 (3), acidocin 8912 from *Lactobacillus acidophilus* TK8912 (8,22), plantaricin A from L*actobacillus plantarum* (18), gassericin A from *Lactobacillus gasseri* LA39 (17) and plantaricin-149 from *Lactobacillus plantarum* NRIC 149 (10) have been detected, purified and characterized. However, nisin is the only bacteriocin being used. Nisin produced by *Lactococcus lactis* is a 3500 Da peptide consisting of unusual amino acids (9). Because of its broad inhibitory spectrum with high antimicrobial activity and its high stability at low pH and high temperatures, nisin is used as a preservative in foods in over 45 countries. It has also been used in health care products and cosmetics for treatment of acne. They are also being used in toothpaste and mouthwash for the inhibition of dental caries and periodontal diseases (5).

Figure 1: Determination of titer of bacteriocin LC-09.



1 = Control 3 = 40 AU/ml 5 = 160 AU/ml 7 = 640 AU/ml 9 = 2560 AU/ml
2 = 20 AU/ml 4 = 80 AU/ml 6 = 320 AU/ml 8 = 1280 AU/ml 10 = 5120 AU/ml

Many heat resistant *Lactobacillus* bacteriocins have been reported such as LP27 (24), lactacin B (1) and enterocin 101 (10) whereas some heat sensitive bacteriocins have also been reported (9). In this paper, we report a novel bacteriocin, produced by an indigenous strain of lactobacilli isolated from infant feces sample. It is heat resistant and active against species other than lactobacilli.

MATERIALS AND METHODS

Bacterial strains and media

Lactobacillus strain LC-09, isolated from a clinical sample (infant feces), was used as a bacteriocin producing strain. All lactobacilli strains used in this study were grown in MRS broth at 37°C for 24 hours and other non-lactic acid bacteria were grown in nutrient broth.

Detection of inhibitory activity

The bacterial strains used as indicator organisms are listed in Table 1. The inhibitory activity of LC-09 was screened by agar well diffusion assay (20). Overnight culture of LC-09 was inoculated in MRS broth and incubated for 24 hours at 37°C. Cells were removed by centrifugation at 10.000 xg for 15 minutes at 4°C. The supernatant fluid was adjusted to pH 7.0 with 4M NaOH. Prepoured MRS agar plates were overlaid with 5 ml MRS soft agar containing 70 μl of indicator cultures. Wells of 5 mm in diameter were cut into the agar plate by using a cork borer and 100 μl of the culture supernatant fluid was placed into each well. Nutrient agar was used for other Gram-positive and Gram-negative indicator cultures. The plates were incubated overnight at 37°C.

Bacteriocin assay

The critical dilution assay described by Mayr-Hartings *et. al.* (15) was used to quantify the inhibitory activity exhibited by the bacteriocin against the respective sensitive indicator strain, ML-01 (*Lactobacillus* strain, isolated from milk sample). A serial two-fold dilution of supernatant fluid, adjusted to pH 7.0, was made in fresh MRS broth. The activity in each dilution was determined by agar-well diffusion method. The titre was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units (AU) per ml.

Effect of enzymes, pH, temperature and detergents

The sensitivity of the bacteriocin produced by LC-09 to different enzymes was checked. Cell-free supernatant fluid at pH 7.0 was treated with trypsin, subtilysin, pronase E and catalase at a final concentration of 0.5 mg/ml. Remaining activity was assayed by agar well diffusion method. To demonstrate the effect of pH, culture supernatant was adjusted to pH values from 1-12 with 4M HCl and 4M NaOH. The activity was then determined by agar well diffusion method. Heat stability of the bacteriocin was assessed by heating the culture supernatant fluid at 80°C and 100°C in water bath. Samples were withdrawn at different time intervals and the activity was checked by the same method. Heat resistance was also checked after autoclaving the bacteriocin at 121°C for 15 minutes. The antagonistic agent of LC-09 was also treated with EDTA, Tween 20, Tween 80, SDS and Triton X-100 at a final concentration of 1%. Agar well diffusion method was used to check the remaining activity of the agent.

Lacuna assay

To determine the number of lacuna forming cells in the culture of LC-09, the cells were exposed to chloroform and were then mixed with sensitive indicator bacteria and soft agar and poured on to the surface of MRS agar plate. Plate was then incubated at 37°C for 24 hours.Lacuna frequency was calculated by the formula:

$$LF = \frac{LFC}{LFC + VBC}$$

LF : Lacuna frequency
LFC : Lacuna forming cell
VBC : Viable bacterial cells

Curing of plasmid

Mutants of LC-09 defective in bacteriocin production were isolated by curing experiments as described by Hirota (6). Acridine orange and ethidium bromide were used as chemical curing agents. The test organism was grown in MRS broth supplemented with acridine orange and ethidium bromide (final concentration 5-150 mg/μL). Both the sets were incubated at 37°C for 24 hours. Culture tube with highest concentration of the dye and showing visible growth was selected and was ten-fold diluted in fresh MRS broth, 500 μL of the dilutions were spread on to MRS agar plates with the help of a sterile spreader. After overnight incubation at 37°C, colonies were checked by agar well diffusion method for bacteriocin production.High temperature and prolonged incubation were used as physical agents to cure the bacteriocinogenic determinants in LC-09.

RESULTS AND DISCUSSION

The inhibitory spectrum of the cell-free supernatant fluid of LC-09 is shown in Table 1. The antibacterial substance produced by LC-09 was found to be inhibitory against some gram-positive bacteria including *Listeria ivanovii, Streptococcus agalactii*, and *Streptococcus pyogenes*. Other gram-positive and gram-negative bacteria tested were not inhibited by the antagonistic agent produced by LC-09.

The titer of lactocin LC-09 against the indicator strain ML-01 was found to be 1280 AU/ml (Figure 1). This activity was comparatively lower than 1350 AU/ml which was recorded in case of *L. acidophilus* JCM 1028 (23) whereas activity lower than lactocin LC-09 was reported in case of plantaricin 149 (9) and gassericin A (12) as 800 AU/ml and 640 AU/ml respectively.

The antagonistic substance produced by LC-09 was completely inactivated by proteases (Table 2), reflecting its proteinaceous nature. Since no effect of catalase on the agent was observed, this ruled out the possibility that inhibitory activity was due to hydrogen peroxide. Certain detergents seem to have a profound effect on the bacteriocin of LC-09. Antagonistic activity of the bacteriocin was greatly reduced when treated with SDS whereas Triton X-100 and Tween 20 completely inhibited the activity. As reported earlier, these agents dissociate the large units of the bacteriocin into the smaller subunits. It can be concluded that the bac-

Table 1: Inhibitory spectrum of the bacteriocin produced by LC-09.

Indicator strain	Sensitivity
Lactobacilli strains	
ML-01	+
ML-02	+
ML-03	-
ML-04	-
ML-05	-
ML-06	±
ML-14	+
Other organisms	
Candida albicans	-
Escherichia coli	-
Listeria ivanovii	+
Listeria monocytogenes	-
Salmonella typhi	-
Salmonella paratyphi A	-
Salmonella paratyphi B	-
Shigella dysentriae	-
Shigella sonnei	-
Staphylococcus albus	-
Staphylococcus aureus	-
Streptococcus agalactii	+
Streptococcus mutans	-
Streptococcus pyogenes	+

Inhibitory activity was determined by agar well diffusion assay.
+ : Zone of inhibition - : No inhibiton

Table 2 : Effects of enzymes, detergents and temperature on the antibacterial activity of the bacteriocin produced by LC-09.

Treatment	Inhibition
Proteases	
Trypsin, subtilysin, pronase E	-
Catalase	+
Detergents (1%)	
SDS	+
Tween 20	-
TX-100	-
Tween 80	+
EDTA	+
Temperature	
100°C, 4 hours	+
121°C, 15 minutes	+
Control	+

+ : Inhibition of the indicator strain.
- : No inhibition.

teriocin of LC-09 is active in state of large units and dispersion of these large active bacteriocin units into small subunits, by these detergents, resulted in the reduction or complete inhibition of antibacterial activity. A similar conclusion was previously made by Tagg *et. al.* (21). In contrast Muriana and Klaenhammer (17) and Kato *et. al.* (10) have reported the bacteriocins which were more active when their large units were dispersed into small active subunits.

Bacteriocins differ greatly with respect to sensitivity to pH. Many of them are considerably more tolerant of acid than alkaline pH values (21). Bacteriocin produced by LC-09 exhibited the same profile and was active at pH values between 3-7. Maximum inhibitory activity was demonstrated at pH 4 and 5.

A novel property of our bacteriocin was its stability at 100°C for four hours, moreover, the agent retained its inhibitory activity even after heating at 121°C for 15 minutes. Although many heat resistant *Lactobacillus* bacteriocins have been reported previously, but heat stability for such a long period of time (4 hours at 100°C) has not been reported. Previous reports are not in accordance to this finding. It seems, we are reporting this for the first time. Previously LP27 (24), lactacin B (1) and enterocin 101 (10) were reported stable at 100°C for 60 minutes. Heat stability of lactocin LC-09 may be due to its complex nature.Lacuna assay has previously been used to determine the number of colicin-producing cells, existing in a culture at one time. Not all bacteriocinogenic strains form lacuna, either because individual cells do not form sufficient bacteriocin to produce a visible clearance or because the bacteriocin remains cell bound and does not diffuse away from individual cells. But in case of LC-09, individual cells produce clear zones and percentage of lacuna forming cells was 0.2%. This proves that LC-09 is a strong bacteriocin producer because individual cells were able to form sufficient bacteriocin that diffused away and produced a visible clearance. Percentage of lacuna forming cells was more than the cultures of *E. coli* containing plasmid Col E_2-P9, in which the percentage of lacuna forming cells was 0.01% (4).

Bacteriocin structural genes have been shown to be either plasmid or chromosome-borne. Our preliminary results indicate that the bacteriocin producing ability of LC-09 might be plasmid mediated, because curing experiments with chemical curing agent led to the obtention of variants which had lost the characteristic of bacteriocin production. Curing at elevated temperature (40°C) also resulted in the complete inhibition of antibacterial activity.The inhibitory spectrum of the lactocin LC-09 is not very broad. Nonetheless, its acid tolerance and stability at high temperature makes the antagonistic agent a tool for exploitation in the field of biotechnology.

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