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Antilisterial Activity of a Broad-Spectrum Bacteriocin, Enterocin LR/6 from *Enterococcus faecium* LR/6

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Abstract Enterocin LR/6, a purified bacteriocin, exhibited broad inhibitory spectrum both against related as well as some food-borne pathogens such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Aeromonas* sp**.,** *Shigella* sp., and *Bacillus licheniformis*. In this investigation, we have focused on *L. monocytogenes* as the target organism, as it is not only an important pathogen but can also survive over a wide range of environmental conditions such as refrigeration temperature, low pH, and high-salt concentration. This allows the pathogen to overcome many food preservation and safety barriers and poses a potential risk to human health. The enterocin LR/6 showed a bactericidal action against *L. monocytogenes* and completely inhibited the growth on agar plates, supplemented with 200 AU/ml of enterocin LR/6. The effectiveness of enterocin LR/6 in completely killing a population of acid-adapted (pH 5.2, 2 h) *L. monocytogenes* exposed to different temperatures (4–37 °C), pH (2.5–8.0), and osmotic (up to 30% NaCl) stress is reported here. This paper focuses on the key issue of killing of the acid-adapted *L. monocytogenes* cells under adverse environmental conditions.

Keywords Acid adaptation · Enterocin LR/6 · Inhibitory spectrum · *Listeria monocytogenes*

Introduction

The antimicrobial proteinaceous molecules referred as bacteriocins, produced by lactic acid bacteria (LAB) and active against taxonomically related bacteria, have been considered as promising biopreservative [1, 2]. Bacteriocins have been classified into four classes (class I, II, III, and IV) based on their genetic and biochemical characteristics [3]. Among the bacteriocins active toward *Listeria,* the lantibiotic nisin has been widely studied and is currently used in many countries as a preservative in food products. Another group of antilisterial peptides produced by some LAB forms a subclass of bacteriocins, the class IIa [1]. Although several bacteriocins from LAB have been characterized to date, their use as

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food preservatives is still not fully realized because of the limited information on target organisms.

Illness caused due to the consumption of contaminated foods has an economic and public health impact worldwide. Moreover, pathogens such as *Listeria monocytogenes* can adapt to survive and grow in a wide range of environmental conditions and cause listeriosis, a severe disease with high hospitalization and case fatality rates [4]. Consumer's demand and serious health implications have stimulated research in the field of biopreservation that refers to the use of antagonistic microorganisms or their metabolic products to inhibit the undesired microorganisms in foods. This is expected to enhance food safety and extend the shelf life [5].

Listeria is a Gram-positive pathogen, with the ability to adapt to a wide range of conditions, generally considered as antimicrobial, such as low temperatures (2–4 °C), acidic, and high-salt conditions [6]. *L. monocytogenes* can be found in a variety of raw and processed foods, milk and dairy products, various meat products, soil, vegetation, water, agricultural and industrial wastes, etc. Among the environmental conditions unfavorable for listerial growth, acidity is probably encountered most commonly, both in natural habitats (acid rain, manure, silage, fermented foods, and feed products) and in infected host systems (gastric secretions, phagocytosomal vacuoles, etc.). The phenomenon of acid tolerance in bacteria has been studied for many years. However, the majority of this research has been directed toward *Escherichia coli* and *Salmonella* [7]. Work on *Listeria* and other Gram-positive bacteria, in comparison, has received far less importance [5, 7–9]. From an application point of view, it is important to know how the specific factors involved in food preservation may affect the sensitivity of the target cells.

We have earlier reported the isolation and purification of an antimicrobial compound, enterocin LR/6 from *Enterococcus faecium* LR/6, with a bactericidal mode of action. Enterocin LR/6 is highly stable over a wide range of pH (2.0 to 8.0) and high temperature (boiling and autoclaving) and could be stored stably at a range of temperature (−4° to 37 °C) at least up to 1 year. Being proteinaceous in nature is being sensitive to many proteolytic enzymes but insensitive to α-amylase, lipase, surfactants, and organic solvents. Tricine SDS-PAGE analysis and bacteriocin activity assay corresponded to a protein of an apparent molecular mass of ~6.0 kDa [10].

The aim of the present study was to determine the inhibitory spectrum of enterocin LR/6 and also describe whether the susceptibility of *L. monocytogenes* changes due to food-relevant stress factors, such as temperature, pH, and salt in a chemically defined medium.

Material and Methods

Bacterial Strains, Culture Media, Growth Conditions, and Chemicals

The bacterium *E. faecium* LR/6 was propagated in optimized TGYE medium at 37 °C and 200 rpm in an incubator shaker (Kuhner, Switzerland) as described earlier [10]. *L. monocytogenes* used as a target organism was grown in nutrient broth (peptone, 5.0 g/l; beef extract, 3.0 g/l; NaCl, 5.0 g/l, pH 7.0) at 30 °C and 200 rpm. Growth was generally monitored turbidometrically in terms of optical density (A_{630}) (Genesys 10vis, Thermospectronic, USA) as well as viable cell counts. All the chemicals were obtained from Sigma-Aldrich, USA, and media components were purchased from Hi-media, Mumbai, India.

Agar Well Diffusion Assay

The antimicrobial activity of the bacteriocin was determined using the agar well diffusion assay method [11]. Aliquots (100 μl) of the enterocin LR/6 were placed in 6-mm-diameter wells that had been cut in 0.8% soft agar plates seeded with the test bacteria (~10^8 CFU/ml). After overnight incubation, the diameter of the zone of growth inhibition was measured.

Determination of Colony-Forming Units

Survival of the target organism was also determined by colony-forming units (CFU; CFU/ml). For this, cell suspension differently treated and serially diluted in sterile normal saline (0.85% NaCl) was plated on nutrient agar. Viable cells were counted after overnight incubation at 30 °C.

Determination of Antimicrobial Spectrum

Enterocin LR/6 was tested for the antimicrobial spectrum against a wide range of bacteria comprising both Gram-positive and Gram-negative members, propagated in appropriate media as listed in Table 1. The inhibition was checked in terms of agar well diffusion assay as well as colony-forming units as described above.

Acid Adaptation of *L. monocytogenes*

L. monocytogenes grown in nutrient broth was harvested from the exponential growth phase. The cells were collected by centrifugation (6,000×g, 10 min), resuspended in 0.85% saline (pH 5.2), and incubated for 2 h at 30 °C [7]. Such cells were plated on nutrient agar, and the surviving cells were labeled as acid-adapted, while the nonadapted cells were obtained without being given such a treatment.

Growth of *L. monocytogenes* on Agar Plates Supplemented with Enterocin LR/6

The plates were poured with the nutrient agar supplemented with 50, 100, and 200 AU/ml of purified enterocin LR/6 [10]. These plates were overlaid individually with acid-adapted and nonadapted *L. monocytogenes* (~10^8 CFU/ml) mixed in the soft agar (0.8%). Plates were incubated overnight at 30 °C, and results were expressed as viable cell counts per milliliter. Plates without enterocin LR/6 were used as control. One bacteriocin unit was arbitrarily defined as the amount of bacteriocin that inhibited the growth of the indicator organism by 50% in comparison to an untreated control.

Kinetics of Cell Destruction by Enterocin LR/6

Approximately 10^8 CFU/ml each of acid-adapted and nonadapted types of *L. monocytogenes* were suspended in sterile normal saline, pH 7.0, containing 200 AU/ml of enterocin LR/6, and incubated at 30 °C. Samples were removed at regular intervals for the determination of residual viable cells by plating the appropriate dilutions on nutrient agar. Viable cells were counted after an overnight incubation at 30 °C. Cell suspensions in normal saline alone were used as respective control.

Table 1 Inhibitory spectrum of enterocin LR/6 (200 AU/ml).

Test organism	Source	Sensitivity
LAB isolates		
LR strains LR/3, 4, 5, 10, 16	Lab stock	+
LR strains LR/1, 14	–	−
Enterococcus casseliflavus NRRL B-3502	NRRL, USA	+
Lactococcus lactis lactis NRRL B-1821	–	+
Lactobacillus pentosus NRRL B-227	–	−
L. acidophilus NRRL B-4495	–	−
L. acidophilus NRRL B-3468	–	−
L. plantarum NRRL B-4496	–	−
L. helveticus NRRL B-4526	–	+
L. casei casei NRRL B-1922	–	−
L. delbrueckii NRRL B-1924	–	−
L. lactis cremoris NRRL B-634	–	−
L. brevis NRRL B-4527	–	+
L. mesenteroides mesenteroides NRRL B-1118	–	−
L. vitulinus NRRL B-14854	–	−
L. sakei NRRL B-1917	–	−
L. delbrueckii NRRL B-763	–	−
Food-borne pathogens		
Yersinia enterocolitica	AIIMS, India	+
Aeromonas sp.	–	+
Listeria monocytogenes (target strain)	–	+
Pseudomonas aeruginosa	–	+
Shigella sp.	–	+
E. coli (urogenic)	–	−
Staphylococcus aureus	–	−
Salmonella enterica	–	−
Bacillus sp.	–	−
Bacillus licheniformis	–	+
Vibrio cholerae	–	−

+ = sensitive; − = resistant

NRRL Northern Regional Research Laboratory, ARS Culture Collection USA; *AIIMS* All India Institute of Medical Sciences, India

Note: All LAB strains were grown in MRS and food-borne pathogens in NB except for *Y. enterocolitica*, which was grown in TSB.

Effect of Enterocin LR/6 on the Acid-adapted and Nonadapted *L. monocytogenes* Under Different Temperatures, pH, and NaCl Concentration

The viability of the acid-adapted and nonadapted *L. monocytogenes* (~10^8 CFU/ml) was checked at different temperatures 4, 10, 30, and 37 °C after overnight incubation. Both types of cells raised at 30 °C were taken as respective controls. In parallel, both types of cultures were also exposed overnight to enterocin LR/6 (200 AU/ml) at these temperatures. Viable cell counts were determined as described above. Untreated samples were taken as control.

Similar populations of two types of cells were incubated for overnight in 0.85% saline set at different pH, 2.5, 4.2, 5.2, 7.0, and 8.0. Samples were removed and plated to determine the viable cell counts. The nonadapted cultures served as respective controls. In another set of experiments, these cell suspensions (~10^8 CFU/ml) were also treated with 200 AU/ml of enterocin LR/6 at different pH, as given above, and viable cell counts were determined. The samples without enterocin LR/6 served as control.

To determine the effect of osmotic shock, the acid-adapted and non-adapted cells of *L. monocytogenes* (~10^8 cells) were exposed to 5%, 10%, 20%, and 30% concentration of NaCl solutions. As before, the cell suspensions of nonadapted culture were taken as control. As described above, such cells were also treated with 200 AU/ml of enterocin LR/6 along with the above-described NaCl concentrations overnight. Cell suspensions without antimicrobial compound were taken as control. The results were expressed by viable cell count per milliliter of the culture.

Statistical Analysis

Each result is expressed as mean along with respective standard error of mean. Each data point is the average of three repeated measurements from two independent replicates.

Results and Discussion

We have isolated and purified enterocin LR/6 from the culture supernatant of *E. faecium* LR/6 [10]. The purified enterocin LR/6 confirmed the antimicrobial spectrum of crude preparation suggesting that enterocin LR/6 is perhaps the only antimicrobial compound produced by *E. faecium* LR/6.

Antimicrobial Spectrum

In order to study the antimicrobial spectrum of enterocin LR/6, several LAB strains and food-borne pathogenic bacteria comprising both Gram-positive and Gram-negative members were tested. As is clear from the results, highly efficient activity was observed against *L. monocytogenes*, *Pseudomonas aeruginosa*, *Aeromonas* sp., *Yersinia enterocolitica*, *Bacillus licheniformis*, *Shigella* sp. as well as against several strains of LAB (Table 1). Several species of *Enterococcus* have been reported to produce bacteriocins; most of which are active only against genetically related species, except for the broad-spectrum Enterocin 012 [12], Enterocin I [13], and Enterocin AS-48 [14]. Enterocin LR/6 can, therefore, be classified under the latter category.

One of the interesting properties of enterocin LR/6 was observed to be its antilisterial action. *L. monocytogenes* is known for its ubiquitous nature, hardiness, and the ability to adapt and grow under a wide range of harsh environmental conditions, such as refrigeration temperature, acidic, and high-salty foods making this well-recognized pathogen a threat to the safety of public health. In other words, any attempt to check the growth of this pathogen using the above-mentioned traditional conditions is not likely to succeed [6]. Bacteriocins have been considered as potential agents, which could check the growth of large number of bacteria [2]. It is, however, important to study the antimicrobial potential of such agents against the selected organisms. For the present investigation, we selected *L. monocytogenes* as the target host

and looked at its response to enterocin LR/6. Acid adaptation in bacteria is reported to provide cross-protection to many other growth inhibitory conditions [7]. We, therefore, worked with acid-adapted *L. monocytogenes* cells and compared them with normal cells (nonadapted) while taking these factors into consideration, especially in their response to enterocin LR/6. To begin with, we looked at the effect of enterocin LR/6 on the growth of *L. monocytogenes*.

Growth of *L. monocytogenes* Strain on Agar Plates Supplemented with Enterocin LR/6

The antilisterial effect was observed at all the concentrations tried, as determined by the agar plate assay. While the effect was clearly concentration-dependent, 200 AU/ml of enterocin LR/6 was able to completely inhibit the growth of *Listeria*. Interestingly, with a viable population of 8.22 $\log_{10}$ CFU/ml, the response of both acid-adapted and nonadapted cells to enterocin LR/6 was similar. Nisin has also been reported to act in the same way [15]. The results of the agar plate assay suggested that enterocin LR/6 could inhibit the proliferation of *L. monocytogenes* in the foods during storage.

Kinetics of Cell Destruction by Enterocin LR/6

As shown in Fig. 1, the incubation of both types of cells (acid-adapted and normal) with enterocin LR/6 suggested bactericidal mode of action. When the killing kinetics was followed over a period of 12 h, enterocin LR/6 could gradually reduce the number of viable cells affecting a complete loss of viability after 10 and 8 h, respectively. The mechanism of this inhibition is under investigation. Acid-adapted cells did face the bacteriocin better in comparison to nonadapted cells but were also eventually killed. A similar mode of action has been observed for enterocins produced by *E. faecium* CCM 4231, RZS C5, and RZS C13 [16]. In contrast, pediocin ST18 from *Pediococcus pentosaceus* ST18 [17] showed bacteriostatic action.

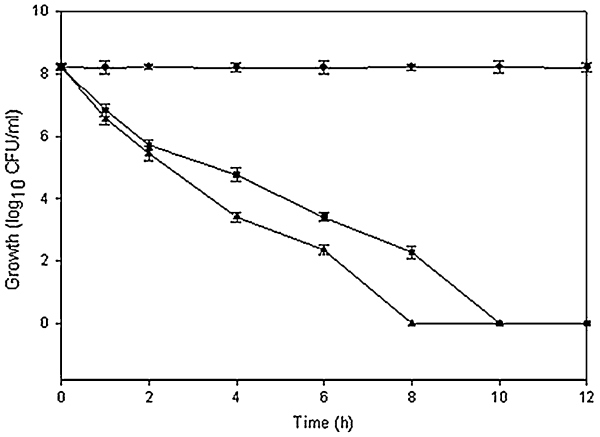

Fig. 1 Nature of antimicrobial action of enterocin LR/6 on indicator strain, *L. monocytogenes*. Control untreated cells (*filled black circle*); acid-adapted (*filled black square*) and nonadapted (*filled black triangle*) cells of *L. monocytogenes* treated with 200 AU/ml of enterocin LR/6

Effect of Enterocin LR/6 Under Different Temperatures, pH, and Concentrations of NaCl on the Acid-Adapted and Nonadapted *L. monocytogenes*

At low temperatures (4 and 10 °C), the acid-adapted cells showed ~30% more viability in comparison to nonadapted cells. However, at temperatures of 30 and 37 °C, both types of cells grew like control (~8.25 $\log_{10}$ CFU/ml). Treatment with enterocin LR/6 led to the complete loss of viability of both cell types at all incubation temperatures, i.e., 4, 10, 30, and 37 °C. While nisin produced from *Lactococcus lactis*, IPLA 1064 is reported to be effective at temperatures of 4, 10, and 32 °C; the bacteriocins from *Lactobacillus paraplantarum* IPLA C23 is ineffective at low temperatures but active at 32 °C [15]. Thus, enterocin LR/6 could be more effective in controlling *Listeria* growth in a range of temperatures.

Acid adaptation is reported to enhance the survival of *L. monocytogenes* in acidified dairy products resulting in greatly improved survival in low-pH foods, like orange juice [8]. In our experiments, the normal *Listeria* cells, in comparison with acid-adapted cells, showed ~40% and 20% reduced viability at pH 2.5 and 4.2, respectively, but at pH 7.0 and 8.0, the viability exhibited by both culture types was comparable. Interestingly, a complete loss of viability was recorded on treatment with 200 AU/ml of enterocin LR/6 of both types of cells irrespective of the pH tested. This is in contrast to the acid-adapted *L. monocytogenes* that has been reported to display enhanced tolerance against the antibiotics, nisin, and lacticin 3147 [18].

The use of salt to lower the water activity is one of the methods of food preservation used by the food industry. However, the ability of *Listeria* to adapt and survive in high concentrations of salt has posed a big challenge. The acid adaptation is known to induce a cross-protection against osmotic stress as well [9]. We have studied the response of acid-adapted and nonadapted *L. monocytogenes* cells to various concentrations of sodium chloride, and the results are shown in Fig. 2. As is clear from the results, the acid-adapted cells could tolerate the increasing salt concentrations better in comparison to nonadapted types. Exposure to enterocin LR/6 (200 AU/ml) could completely eliminate the cells of *L. monocytogenes* at all the concentrations of NaCl

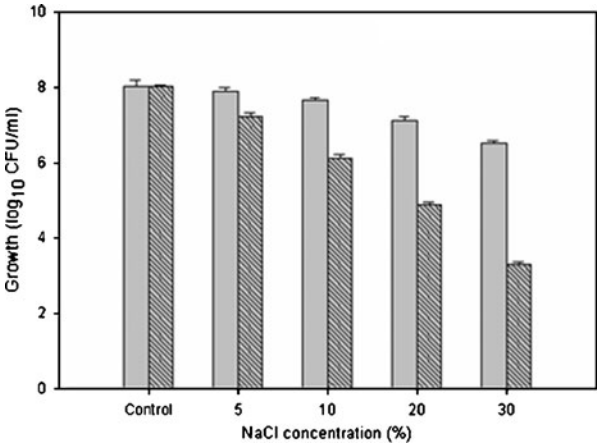

Fig. 2 Effect of acid adaptation on the viability of *L. monocytogenes* at different concentrations of NaCl. Nonadapted control (*diagonal shaded bar*) and acid-adapted cells (*grey bar*). Note: Under all conditions, enterocin LR/6 (200 AU/ml) could completely inhibit the growth of *L. monocytogenes*

irrespective of their acid adaptation status. In comparison, 6.5% NaCl treatment is known to make the pediocin from *Pediococcus acidilactici* PA-2 completely ineffective [19].

The cross-resistance of acid-adapted cells to other stresses has important implications for the food industry, particularly since foods commonly encounter sublethal acidic treatments during various stages of processing [18]. Our results have clearly demonstrated that the survival of *L. monocytogenes* in the presence of high salt or at different pH and temperature can be completely checked by enterocin LR/6. We thus propose that enterocin LR/6 possesses all the properties to serve as an effective food preservative.

Conclusions

Enterocin LR/6, a bacteriocin, has been purified from the culture filtrate of *E. faecium* LR/6. When tested for antimicrobial activity, it exhibited broad inhibitory spectrum both against related as well as some food-borne pathogens, such as *L. monocytogenes*, *Y. enterocolitica*, *Aeromonas* sp., *Shigella* sp., and *B. licheniformis*. It completely inhibited the growth of *L. monocytogenes* on agar plates, supplemented with 200 AU/ml of enterocin LR/6, and showed a bactericidal action against *L. monocytogenes*. The enterocin LR/6 completely eliminated the population of acid-adapted *L. monocytogenes* exposed to different temperatures (4–37 °C), pH (2.5–8.0), and osmotic (up to 30% NaCl) stress. The killing of the acid-adapted *L. monocytogenes* cells under adverse environmental conditions provides a solution for the control of this important food-borne pathogen.

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