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Morphological Changes Induced in *Listeria monocytogenes* V7 by a Bacteriocin Produced by *Pediococcus acidilactici*

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Abstract *Pediococcus acidilactici* produces bacteriocin, which kills *Listeria monocytogenes*. The bactericidal mode of action of the bacteriocin against *L. monocytogenes* V7 was investigated by transmission electron microscopy. The bacteriocin was purified partially from the cell-free extract using Micro-Cel and cation-exchange chromatography, and the specific activity was increased 1,791 fold. The bacteriocin (6,400 AU/ml) was inoculated with *L. monocytogenes* V7 and incubated for 0.5 h, 1 h, 3 h, and 6 h. The bacteriocin was found to destroy most of the cell wall and released most of the inclusions in the cells after 6 h of incubation. These results suggest that the bactericidal effect of the bacteriocin was due to bacterial lysis.

Keywords: Bacteriocin, *Pediococcus acidilactici*, *Listeria monocytogenes*, transmission electron microscopy

Many bacteriocins from Gram-positive bacteria have fairly broad inhibitory spectra, and these bacteriocins have been attractive as natural food preservatives [2, 10, 13]. Substances currently named bacteriocins are antimicrobial proteinaceous compounds that are lethal to closely related bacteriocin-producing species, foodborne pathogens, and spoilage bacteria, including *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens*, and so on [12, 19]. An understanding of the mechanisms of action of these bacteriocins against susceptible bacterial strains would be important for their effective use in food preservation. The primary target of nisin has been reported to be the cytoplasmic membrane, since treatment with nisin causes rapid, nonspecific efflux of amino acids and cations, collapse of the membrane potential, and cell death [21]. Lactocin from *Lactobacillus helveticus* inhibits protein synthesis, causes cytoplasmic membrane damage, and is bacteriostatic [22]. Pediocin produced by *Pediococcus acidilactici* belongs to a major subgroup of class II bacteriocins. Members of this class have a number of common features, including very strong antimicrobial activity against *Listeria* species [1, 17]. Pediocin from *P. acidilactici* absorbed to a sensitive strain is bactericidal and causes lysis of a strain of *L. monocytogenes* [9]. The foodborne pathogen *L. monocytogenes* is a major concern in the food industry, since it can grow in a variety of food products at low temperature and pH [3, 7, 11, 18]. Because of bacteriocin's antimicrobial activity, many researchers have extensively evaluated bacteriocin for the ability to kill or control pathogens including *L. monocytogenes* [14, 16, 20]. However, the roles of bacteriocin in killing and controlling pathogens are still not fully understood. The objective of this study was to investigate morphological changes induced in *L. monocytogenes* V7 by partially purified bacteriocin, using transmission electron microscopy (TEM).

Materials and Methods

Bacterial Strains and Cultures

L. monocytogenes V7 (source: USFDA), *Lactobacillus plantarum*, and *P. acidilactici* were obtained from the Department of Food Science, University of Arkansas at Fayetteville, U.S.A. Throughout the experiments, strains were subcultured every month on strain preservation agar slants and kept at 4°C. Before experimental use, *L. monocytogenes* was grown in BHI broth at 37°C for 24 h, whereas *P. acidilactici* and *L. plantarum* were grown in MRS broth at 30°C and 37°C for 24 h, respectively.

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Table 1. Steps for bacteriocin purification.

Purification stage	Volume (ml)	Total protein (mg)	Activity (AU/ml)	Total activity (AU)	Specific activity (AU/mg)	Purification fold
CFE[a]	1,500	19,155	3,200	4,800,000	251	1
MC[b]	15	135	225,400	3,381,000	25,044	100
CMC[c]	1.5	1.8	539,361	809,042	449,468	1,791

[a]Cell-free extract.
[b]Micro-Cel.
[c]Cation-exchange chromatography.

Preparation of Crude Bacteriocin

P. acidilactici was inoculated into MRS broth. After 24 h of incubation at 30°C, culture supernatant fluid was collected by centrifugation at 3,000 ×*g* for 5 min. The supernatant fluid was passed through sterile filters (0.22 µm pore size) and lyophilized.

Purification of Bacteriocin

The bacteriocin was purified using Micro-Cel and cation-exchange chromatography (CMC) [24]. Micro-Cel (diatomate calcium silicate; Celite Corporation, Lompoc, CA, U.S.A.) was used for adsorption of bacteriocin. After the lyophilized powder had been reconstituted with distilled water, bacteriocin preparations were adjusted to pH 2.0–9.0 with 5 M phosphoric acid. Micro-Cel was added to yield 2% (w/v). The samples were stirred overnight at 4°C and centrifuged at 1,000 ×*g* for 20 min. Bacteriocin-adsorbed Micro-Cel was washed with distilled water and resuspended with 0.1 M NaCl. The pH of the Micro-Cel samples was adjusted to 2.0 to desorb the bacteriocin. The samples were stirred for 2 h at 4°C and heated at 80°C for 1 min. After centrifugation at 1,000 ×*g* for 20 min, the pH of the supernatant was adjusted to 5.0. The bacteriocin activity was determined during the steps of purification.

The bacteriocin prepared by Micro-Cel was applied to a Whatman carboxymethyl cellulose column (CM-52; W. & R. Balston Ltd., Maidstone, U.K.) previously equilibrated with 50 mM phosphate buffer at pH 6.6. The column (30 cm×2.5 cm) was then washed with the same buffer, followed by a 500-ml linear NaCl gradient (0–1 Mol) in the same buffer. Fractions containing bacteriocin activity were pooled, dialyzed, and lyophilized for further studies.

Determination of Bacteriocin Activity

Antimicrobial activity of the bacteriocin was performed by the agar spot method with a slight modification [20]. Bacteriocin preparations were serially twofold diluted and 5 µl of dilutions was spotted on MRS agar (1.5% agar) plates overlaid with the indicator strain (*L. plantarum*) in 5 ml of soft MRS agar (0.8% agar). These plates were incubated at 37°C for 24 h and examined for zones of inhibition. The bacteriocin activity, expressed as arbitrary units per millilter (AU/ml), was calculated as the reciprocal of the highest dilution of a bacteriocin that gave a minimum of 2-mm diameter zones of inhibition on a lawn of indicator strain.

The bacteriocin (6,400 AU/ml) was added to the culture of *L. monocytogenes* V7 that had been grown for 24 h. After treatment with the bacteriocin, cells were incubated at 37°C for 0.5 h, 1 h, 3 h, and 6 h. The cells were then centrifuged at 5,000 ×*g* for 15 min. The pellets were resuspended in 20 mM phosphate-buffered saline (PBS) at pH 7.0 to original volumes, serially diluted, and then pour-plated with suitable agar medium. After the plates were incubated at 37°C for 48 h, the colony-forming units were enumerated and the percentage of cell death was determined in relation to the control.

Transmission Electron Microscopy

The bacteriocin treated and non-treated cells of *L. monocytogenes* V7 and *P. acidilactici* were prepared by the method described by Zahller and Stewart [25] with slight modification and examined using a transmission electron microscope (JEOL-100 CX, Japan).

Results and Discussion

The activity of this bacteriocin preparation, determined by the agar spot method, was 6,400 AU/ml. A similar pediocin produced by *L. plantarum* WHE 92 isolated from cheese yielded a stronger activity of 17,070 AU/ml [6]. However,

Table 2. Effect of bacteriocin exposure time on the survival of *Listeria monocytogenes* in BHI broth.

Time (h)	CFU/ml	% of death
0	9×10^{10}	–
0.5	9.6×10^{8}	98.9%
1	4.5×10^{8}	99.5%
3	1.99×10^{6}	99.997%
6	4.8×10^{5}	99.999%

The bacteriocin at 6,400 AU/ml was added to the culture of *L. monocytogenes*. All data are means of three separate experiments and significantly different from the data of the control at a P value of <0.05.

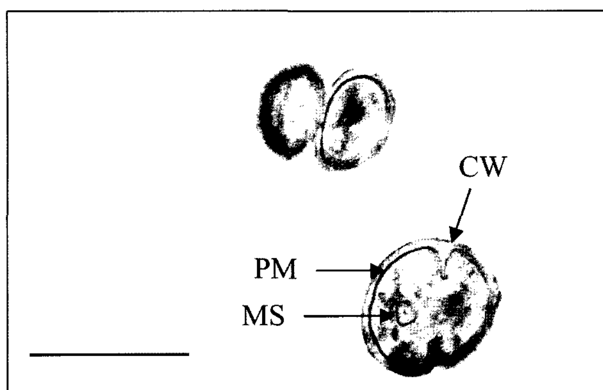

Fig. 1. Transmission electron micrograph of *Pediococcus acidilactici* strains.
CW: cell wall; PM: plasma membrane; MS: mesosome; Scale bar, 2 μm.

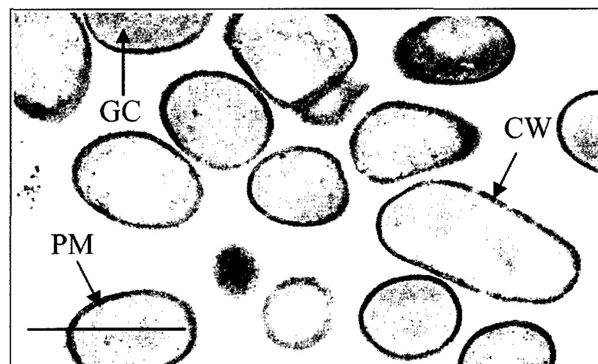

Fig. 2. Transmission electron micrograph of bacteriocin-nor-treated *Listeria monocytogenes* strains.
GC: granular cytoplasm; CW: cell wall; PM: plasma membrane; Scale bar, 2 μm.

the activity of bacteriocin used was stronger than the activity of related lactococcin R, which was 1,600 AU/ml [23]. Thus, the activity of bacteriocin appears to vary, depending on the producing organism growth conditions and the kind of bacteriocin produced.

About 1.8 mg of bacteriocin was purified from 1,500 ml of cell-free supernatant fluid, and Table 1 summarizes the purification steps of the bacteriocin. The specific activity was increased 1,791 fold using Micro-Cel and CMC. This result was similar to that of the bifidocin B produced by *B. bifidum* NCFB [24].

Addition of bacteriocin at 6,400 AU/ml to a culture of *L. monocytogenes* grown for 24 h in BHI broth resulted in reduction of colony counts (Table 2). After 1 h of treatment with the bacteriocin (6,400 AU/ml), more than 99% of the cells were killed.

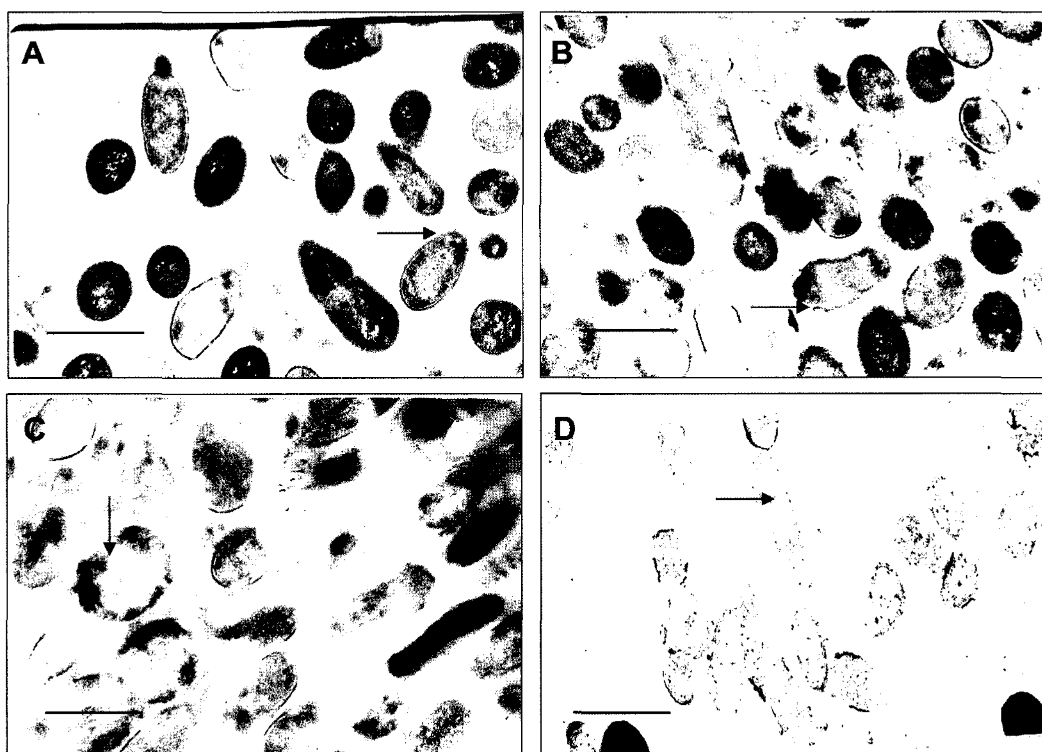

Fig. 3. Transmission electron micrographs of bacteriocin-treated *Listeria monocytogenes* strains.
A. Bacteriocin-treated *Listeria monocytogenes* for 0.5 h; the arrow indicates a relaxed cell wall. **B.** Bacteriocin-treated *Listeria monocytogenes* for 1 h; the arrow indicates an irregular and perforated surface. **C.** Bacteriocin-treated *Listeria monocytogenes* for 3 h; the arrow indicates less cell contents. **D.** Bacteriocin-treated *Listeria monocytogenes* for 6 h; the arrow indicates a ghost cell. Scale bar, 2 μm.

The partially purified bacteriocin (6,400 AU/ml) and pellet of *L. monocytogenes* were mixed and then incubated at 37°C for 0.5 h, 1 h, 3 h, and 6 h to investigate morphological changes of *L. monocytogenes* V7 by TEM. *P. acidilactici*, the bacteriocin-producing bacterium, had a mesosome (MS) (Fig. 1). The mesosome is part of the plasma membrane (PM) and may be naturally synthesized by the cell or formed from a damaged PM. It occurs in various forms: tubular, tubulovesicular, whorl, and a complex wavy anastomosing pattern in the cytoplasma [8].

Control cells of *L. monocytogenes* V7 had a thick and dense cell wall, making it difficult to distinguish between the cell wall and the plasma membrane, since there was no visible space between the two, and cells remained intact (Fig. 2). Conversely, bacteriocin-treated *L. monocytogenes* V7 were almost completely destroyed after 6 h. The major morphological changes were apparently due to changes in the cell wall (Fig. 3). The cell wall started to be relaxed and ruptured after just 0.5 h of treatment (Fig. 3A) with bacteriocin (6,400 AU/ml). After 1 h and 3 h of treatment, ruptures in the cell wall and plasma membrane became more evident (Figs. 3B, 3C), with more cell contents escaping. After 6 h of treatment, the cell wall was completely irregular and damaged (Fig. 3D). For comparisons, *L. monocytogenes* treated with a preparation of pediocin produced by a strain of *L. plantarum* retained a similar appearance after incubation for 0.5 h up to 5 h [6]. The effects on cells due to long-term chilled storage, freeze/thaw, enzyme treatment with lipase or lysozyme, or high hydrostatic pressure were similar to the effects caused by bacteriocins on *Listeria monocytogenes* [4, 5, 15].

In conclusion, the partially purified bacteriocin strongly affected *L. monocytogenes* V7, and TEM of *L. monocytogenes* V7 cells treated with bacteriocin revealed that the bactericidal effect of the bacteriocin was accompanied by cell lysis.

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