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Inhibition of *Listeria monocytogenes* by Using Bacteriocin PA-1 Produced by *Pediococcus acidilactici* PAC 1.0

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The bacteriocin produced by *Pediococcus acidilactici* PAC 1.0, previously designated PA-1 bacteriocin, was found to be inhibitory and bactericidal for *Listeria monocytogenes*. A dried powder prepared from PAC 1.0 culture supernatant fortified with 10% milk powder was found to contain bacteriocin activity. An MIC against *L. monocytogenes* and lytic effects in broth cultures were determined. Inhibition by PA-1 powder occurred over the pH range 5.5 to 7.0 and at both 4 and 32°C. In addition, inhibition of *L. monocytogenes* was demonstrated in several food systems including dressed cottage cheese, half-and-half cream, and cheese sauce.

Listeria monocytogenes is a gram-positive rod that is catalase positive and shows a characteristic tumbling mobility. It has long been recognized as a veterinary pathogen and in humans causes a disease known as listeriosis particularly in neonates or immunocompromised hosts (11). *L. monocytogenes* is found in soil, in decaying and dead vegetation, and in the intestinal tracts of over 50 domestic and wild species of birds and animals including sheep, cattle, chickens, and swine (5). Before 1967, listeriosis was considered a rare disease in healthy adult humans, but recent food-borne outbreaks involving *L. monocytogenes* have highlighted its public health importance (8, 15). Foods such as milk, cole slaw, and Mexican-style cheese were recently involved in listeriosis outbreaks (2, 11, 16). A recall of Brie cheese because of *L. monocytogenes* contamination occurred in 1986 (8). The unique characteristic that makes *L. monocytogenes* a significant threat as a food-borne infection is its ability to grow rapidly at refrigeration temperatures. McLaughlin (11) reviewed the literature on the various ways *L. monocytogenes* could be transmitted through food.

Currently, the procedures recommended for avoiding contamination of foods by *L. monocytogenes* are following sanitary procedures, proper heat treatment, and avoiding postpasteurization contamination (4, 8). Recently, Gonzalez and Kunka (6) described a bacteriocin designated pediocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0 that showed inhibitory activity against a wide spectrum of gram-positive lactic acid bacteria. Although the bacteriocin failed to show any activity against lactic streptococci, *Staphylococcus* spp., and *Micrococcus* spp., because of the fairly wide spectrum of activity against gram-positive genera found in foods, its effect against *L. monocytogenes* was examined. In this paper we describe studies on the inhibitory effect of pediocin PA-1 against *L. monocytogenes*. Definite inhibitory activity was demonstrated against *L. monocytogenes* in broth and agar plate systems as well as in food systems.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are from the Microlife Technics strain collection. *L. monocytogenes*, *P. acidilactici*, and *Pediococcus pentosaceus* were routinely maintained on APT agar (Difco Laboratories, Detroit, Mich.). Isolation of *L. monocytogenes* from foods was done on McBride agar (Difco), on which colonies produced the typical bluish-gray color (10). MIC determinations and growth inhibition and lysis experiments were carried out in APT broth. Dried bacteriocin powder was prepared from cultures grown in supplemented MRS broth (Difco). Nutritional supplements used were yeast extract (Oxoid, Basingstoke, England), Hy-case (Sheffield Products, Norwich, N.Y.), Hy-soy (Sheffield), and Casamino Acids (Difco). Nonfat dry milk powder was obtained from Riley Dairy, Tampa, Fla.

Effect of nutritional supplements on bacteriocin production. *P. acidilactici* PAC 1.0 was inoculated into 100 ml of MRS broth with and without nutritional supplements and incubated at 35°C for 18 h. Then 25 ml of each of the media were centrifuged at 24,000 × *g* for 15 min at 4°C. The supernatants were then filter sterilized with a 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.) and tested against the indicator strain *P. pentosaceus* FBB63.

Production of dried bacteriocin PA-1. *P. acidilactici* PAC 1.0 was grown overnight at 35°C in 1 liter of MRS broth supplemented with 2% yeast extract. The cells were pelleted by centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, Calif.) at 10,000 × *g*, and the supernatant was collected. Nonfat dry milk powder was added to 10% (wt/vol), and the mixture was lyophilized into a dry powder.

Bacteriocin sensitivity determination. Sensitivity of *L. monocytogenes* to PA-1 was determined by inoculating 8-mm (diameter) patches of PAC 1.0 to the surface of an APT agar plate. Plates were incubated at 35°C for 18 h and then overlaid with APT soft agar (0.75%) containing the *Listeria* strain of interest. Overlaid plates were incubated at 35°C for 18 h. At this time, zones of inhibition were measured. To rule out acidic inhibition, sensitivities were confirmed by using APT agar containing 2% sodium beta-glycerol phosphate (Sigma Chemical Co., St. Louis, Mo.) as a buffering agent.

Bacteriocin assay. Samples for determination of bacteriocin activity were filter sterilized with a 0.22-μm-pore-size filter (Millipore). Serial twofold dilutions of the filtrate were made in sterile water. From each dilution, 5 μl was delivered to an MRS soft agar overlay (0.75%) seeded with the sensitive indicator strain *P. pentosaceus* FBB63. Assay plates were incubated at 35°C for 18 h. One arbitrary unit (AU) was defined as 5 μl of the highest dilution of filtrate yielding a definite zone of growth inhibition on the indicator

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lawn. The titer was expressed as the reciprocal of the highest dilution showing inhibition (6).

Determination of the MICs. Strains were grown in APT broth until the exponential phase. PA-1 bacteriocin powder was dissolved in APT broth at an initial concentration of 1,000 AU/ml and filter sterilized. Serial twofold dilutions were made in 1.0 ml of APT broth down to 2.0 AU/ml. Approximately 10^3 bacteria were added to the PA-1 dilutions, and the tubes were incubated for 24 h at 32 or 35°C. The MIC was recorded as the lowest concentration of PA-1 bacteriocin that resulted in no visible turbidity after 24 h of incubation.

Lysis of *L. monocytogenes* by PA-1 bacteriocin. PA-1 bacteriocin powder was added to exponential-phase APT broth cultures of *L. monocytogenes* at 200 or 500 AU/ml, and the tubes were incubated at 32°C. Optical densities were followed at 660 nm on a Spectronic 20 spectrometer (Bausch & Lomb, Inc., Rochester, N.Y.) over several hours. Rates of lysis were calculated by using a variation of the instantaneous-growth-rate equation (12) according to the formula $k = \ln 2/T_D$, where k is the lysis rate constant and T_D represents the time required to reduce turbidity by one-half.

Inhibition of *L. monocytogenes* by bacteriocin PA-1 at different pHs and temperatures. APT broth was adjusted to pH 5.5, 6.0, 6.5, or 7.0 by the addition of 85% lactic acid (Mallinckrodt, Inc., Paris, Ky.) and inoculated with *L. monocytogenes* LMO1 at the indicated initial inoculum. PA-1 powder was added to one series of tubes to provide 500 AU/ml, and an identical set was maintained as a control. Tubes were incubated at the desired temperature and samples were taken at various times, diluted, and plated on APT agar to determine remaining viable CFU per milliliter.

Inhibition of *L. monocytogenes* by pediocin PA-1 in creamed cottage cheese. Dry cottage cheese curd was obtained from a commercial source and was washed once in cold chlorine (approximately 10 ppm) water and allowed to drain thoroughly. Cold, pasteurized, homogenized half-and-half cream from a commercial outlet was added to the washed curd at a 66/34 (wt/wt) ratio of dry curd to cold half-and-half cream. The cream and the curd were thoroughly mixed, and 300-g portions of dressed curd were weighed separately into three plastic cartons that had been sanitized with chlorinated water. The pH of the dressed curd was 5.1. No salt was added to the dressing.

One container (A) was inoculated with an overnight culture of *L. monocytogenes* to give between 10^3 and 10^4 CFU/g of dressed cottage cheese, thoroughly mixed, and placed at 4°C. The second (B) and third (C) containers were inoculated with *L. monocytogenes* at the same rate as A. After mixing, PA-1 powder was added to B to give 10 AU/g of cheese, mixed thoroughly, and refrigerated at 4°C. To container C, PA-1 powder was added at the rate of 50 AU/g cheese mixed well and held at 4°C. After 24 h of incubation at 4°C, the cartons were mixed, and 11.0-g samples were directly weighed into 99.0-ml dilution blanks to make a 1:10 dilution. Further dilutions were made and plated on McBride agar. Plates were incubated at 32°C for 48 h, and typical light blue *L. monocytogenes* colonies were counted. After sampling, the cottage cheese cartons were again refrigerated at 4°C, held for 7 days, and again sampled for growth of *L. monocytogenes*.

Inhibition of *L. monocytogenes* by pediocin PA-1 in half-and-half cream. *L. monocytogenes* grown for 5 h at 32°C in APT broth was added to commercially available cold, pasteurized, homogenized half-and-half cream (18% milk fat, pH 6.6) to provide between 10^2 and 10^3 CFU/ml. The inoculated cream was divided into two equal portions. To one, bacteriocin PA-1 was added to provide 100 AU/ml, and the second portion served as the inoculated control. All the samples were held at 4°C. Counts of *L. monocytogenes* were determined immediately after the experimental protocol was completed. After sampling, the mixtures were refrigerated at 4°C and sampled for the growth of *L. monocytogenes* after 1, 7, and 14 days.

Inhibition of *L. monocytogenes* by pediocin PA-1 in cheese sauce. Canned, condensed Cheddar cheese soup (Campbell Soup Co., Camden, N.J.) was obtained and reconstituted according to the manufacturer's instructions. The pH of the reconstituted soup was adjusted to 6.0 with 1 N NaOH; the soup was divided into equal portions in four separate flasks, heat treated at 100°C for 60 min, and cooled to 4°C in an ice bath. After cooling, two flasks were inoculated with an exponential-phase culture of *L. monocytogenes* to provide about 10^3 CFU/ml of the cheese sauce. To one of the inoculated flasks, bacteriocin PA-1 was added to give 100 AU/ml. Counts of *L. monocytogenes* were determined immediately after the experimental protocol was completed. After sampling, the mixtures were refrigerated at 4°C and sampled for the growth of *L. monocytogenes* after 1, 7, and 14 days. The salt content of the cheese sauce determined by the Association of Official Analytical Chemists titrimetric procedure (1) was 1.0%.

RESULTS

Inhibition of *L. monocytogenes* by *P. acidilactici* PAC 1.0. *P. acidilactici* PAC 1.0 was assayed for inhibition of *L. monocytogenes* by the method of Gonzalez and Kunka (6). Eight strains, seven originally isolated from meat products and one from milk, were tested; all displayed approximately equal (18- to 21-mm) zones of inhibition when used as indicator overlays (data not shown). Inhibitory activity was found with both live cells and cell-free supernatants of PAC 1.0 in buffered and nonbuffered media. When *P. acidilactici* PAC 1.14, a derivative of PAC 1.0 cured of the plasmid encoding PA-1, was used, no inhibition of *L. monocytogenes* was observed by either cells or cell-free supernatants. Since all eight strains showed approximately equal activity, one strain, *L. monocytogenes* LMO1, was chosen for further study.

PA-1 bacteriocin powder was prepared from *P. acidilactici* PAC 1.0 as described in Materials and Methods. PAC 1.0 was grown in MRS broth supplemented with 2% yeast extract, which elicited maximal bacteriocin production. Other nutritional supplements were tested and found not to be as effective as the yeast extract. The resulting dry powder, assayed as previously described (6), contained approximately 16,000 AU/g of powder.

MICs. MICs were determined for *L. monocytogenes* LMO1 and *P. pentosaceus* FBB63, a strain previously used as a sensitive indicator of PA-1 bacteriocin activity (6). MICs averaged for four independent trials were 54.7 AU/ml for LMO1 and 25.4 AU/ml for FBB63.

Lysis of *L. monocytogenes* by PA-1 bacteriocin. PA-1 bacteriocin powder was added to exponential-phase cultures of *L. monocytogenes* LMO1 to examine the effect on growth over time. PA-1 powder was added at a final concentration of either 200 or 500 AU/ml (Fig. 1A), and A_{660} was followed over several hours. The results showed a decrease in turbidity over time indicative of cellular lysis. Rates of lysis were calculated by using the times required to reduce turbidity by one-half. Lysis rates for LMO1 were 0.19/h for 200 AU/ml

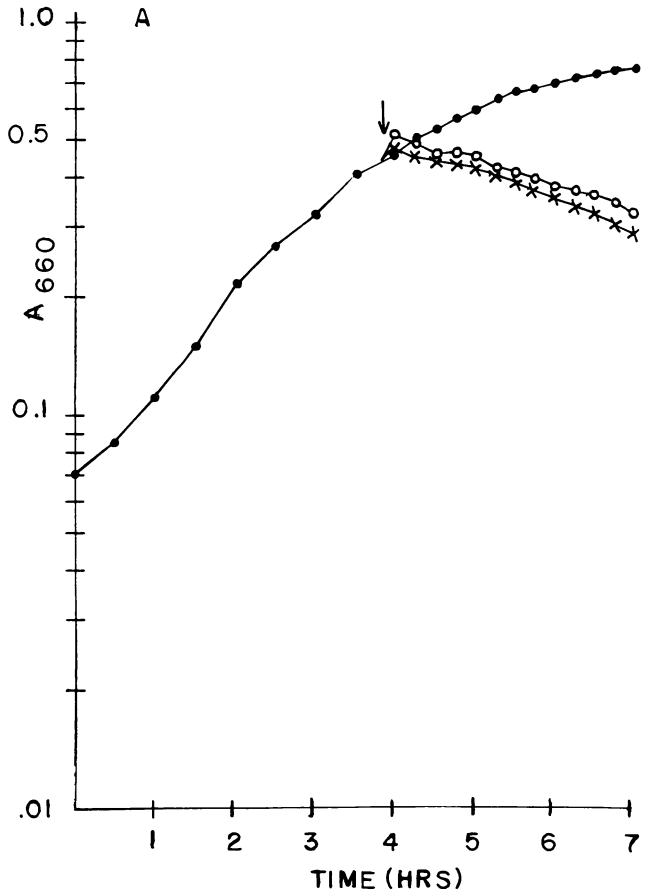

FIG. 1. Effect of PA-1 bacteriocin on the growth of *L. monocytogenes* in APT broth at 32°C. PA-1 bacteriocin powder was added, and turbidity was followed over time at 660 nm. Symbols: ●, control (no PA-1); ○, 200 AU of PA-1 per ml; ×, 500 AU of PA-1 per ml. The arrow indicates the time of PA-1 addition.

and 0.17/h for 500 AU/ml. This compared with a lysis rate of 0.29/h for *P. pentosaceus* FBB63 at 200 AU of PA-1 per ml (data not shown).

Effects of pH and temperature on PA-1 inhibition of *L. monocytogenes*. Inhibition of *L. monocytogenes* by PA-1 at various pHs was determined at both 4 and 32°C. APT broth adjusted to pH 5.5, 6.0, 6.5, or 7.0 was inoculated with *L. monocytogenes* LMO1 at 1.2×10^2 CFU/ml. For tubes held at 4°C, bacterial counts were obtained after 2, 7, and 21 days (Table 1). Bacterial counts in the controls reached over 10^9 CFU/ml after 21 days at pH 6.5 and pH 7.0. At pH 5.5 and pH 6.0, cell numbers increased only slightly. In the matching series of tubes containing PA-1 bacteriocin, no CFU were found at the 10^{-1} dilution from 2 days through 21 days.

At 32°C, bacterial counts were also determined at 2, 7, and 21 days. The counts in the control tubes reached about 10^{10} CFU/ml by 48 h at all four pHs examined and then began to decline. By 7 days, no colonies were found at the 10^{-1} dilution at pH 6.5 and 7.0 and after 21 days, no colonies were found at 10^{-1} dilution at all four pHs. In the matching series of tubes with PA-1, no colonies could be recovered at the 10^{-1} dilution at all four pHs from day 2 through 21 days.

Inhibition of *L. monocytogenes* in refrigerated food systems by bacteriocin PA-1. Table 2 summarizes the results obtained with creamed cottage cheese, half-and-half cream, and cheese sauce. The three food systems were selected to represent high-moisture foods, i.e., water activity of ≥0.95. Cottage cheese was chosen to represent a relatively acid environment with a pH of 5.1. Cheese sauce was selected to represent a mildly acidic system (pH 6.0) with high protein and fat content and 1.0% added salt. Half-and-half cream represented a high-fat (18% milkfat), neutral (pH 6.6) food system. The level of *L. monocytogenes* added to the food systems (10^2 to 10^4 CFU/g or ml) was selected to represent what would probably occur as a postprocessing contamination.

TABLE 1. Effects of temperature and pH on inhibition of *L. monocytogenes* LMO1 by bacteriocin PA-1 in APT broth[a]

Temp (°C)	Time of sample (days)	pH	CFU/ml	
			Control	With PA-1
4	2	5.5	3.1×10^2	<10
		6.0	2.4×10^2	<10
		6.5	2.9×10^2	<10
		7.0	7.8×10^2	<10
	7	5.5	2.2×10^2	<10
		6.0	2.2×10^2	<10
		6.5	1.0×10^3	<10
		7.0	9.7×10^4	<10
	21	5.5	2.5×10^2	<10
		6.0	1.4×10^3	<10
		6.5	3.0×10^9	<10
		7.0	2.2×10^9	<10
32	2	5.5	9.4×10^9	<10
		6.0	1.3×10^{10}	<10
		6.5	1.6×10^{10}	<10
		7.0	1.8×10^{10}	<10
	4	5.5	2.5×10^9	<10
		6.0	2.2×10^9	<10
		6.5	1.8×10^9	<10
		7.0	5.0×10^9	<10

[a] An initial inoculum of 1.2×10^2 bacteria/ml was used. PA-1 was added at 500 AU/ml. At 32°C, no viable colonies were observed at any pH after 21 days in the controls.

TABLE 2. Effect of bacteriocin PA-1 on *L. monocytogenes* added to food systems held at 4°C[a]

Food system	Sampling time (days)	CFU/ml of *L. monocytogenes* in:		
		A	B	C
Cottage cheese	1	7.5×10^3	$<10^{2b}$	$<10^2$
	7	1.0×10^2	$<10^2$	$<10^2$
Half-and-half cream	0	3.0×10^2	$<10^2$	NA[c]
	1	1.6×10^4	$<10^2$	NA
	7	5.4×10^6	5.9×10^3	NA
	14	2.0×10^8	9.0×10^5	NA
Cheese sauce	0	7.0×10^2	$<10^2$	NA
	1	2.6×10^3	$<10^2$	NA
	7	1.7×10^7	1.0×10^2	NA
	14	2.0×10^8	3.0×10^4	NA

[a] A, Inoculated control, no PA-1 added; B, inoculated and PA-1 added at 10 AU/g for cottage cheese and 100 AU/ml for half-and-half cream and cheese sauce; C, inoculated and PA-1 added at 50 AU/g for cottage cheese.

[b] Represents lower limit of detection.

[c] NA, Not applicable for half-and-half cream and cheese sauce.

With the exception of cottage cheese, which had a relatively low pH of 5.1, the other two systems allowed rapid growth of the pathogen at 4°C. There was approximately a 10^6-fold increase in counts over a 14-day period in both the half-and-half cream and cheese sauce. In the cottage cheese system, the initial count decreased by a factor of 10 over a 7-day period. In all of the food systems, there was a rapid decrease in viable counts of *L. monocytogenes* in the presence of bacteriocin PA-1. There was, however, a resurgence of the pathogen after 7 days of holding at 4°C in the mildly acidic and neutral food systems. In the acidic cottage cheese system, however, the viable numbers of *L. monocytogenes* were held in check after 7 days at 4°C in the presence of bacteriocin PA-1 even at 1/10 the concentration of the bacteriocin added to half-and-half cream and cheese sauce.

DISCUSSION

The results presented here describe the activity of a bacteriocin produced by *P. acidilactici* PAC 1.0, first described by Gonzalez and Kunka (6), against *L. monocytogenes*. Eight *L. monocytogenes* isolates were screened by plate assay, and all were sensitive to the PAC 1.0 bacteriocin, PA-1. The observed zones of inhibition were not due to acid because of the large size (18 to 21 mm) of the zones and their sharp, distinct borders, the absence of such zones with the cured derivative of PAC 1.0, and the presence of the zones around PAC 1.0 when buffered MRS medium was used.

The MIC was 54.7 AU of PA-1 per ml for *L. monocytogenes*. This compared with 25.4 AU/ml for *P. pentosaceus* FBB63, a strain previously used as a sensitive indicator for PA-1 bacteriocin activity (6). These values occurred at initial inocula of 10^3 CFU/ml. There was a cell concentration effect; larger initial indicator inocula resulted in higher MIC values. The effects of PA-1 bacteriocin on growing *L. monocytogenes* cultures over time indicated a loss of turbidity over time. Lysis rates were virtually identical for both concentrations of PA-1 against *L. monocytogenes* but less than the rate for *P. pentosaceus* FBB63, which correlates with the lower MIC value for the pediococci. These data indicate that *L. monocytogenes* is sensitive to the effects of PA-1, although slightly less than *P. pentosaceus* FBB63.

The effects of pH and temperature on PA-1 inhibition of *L. monocytogenes* were examined. Since the organism survives and grows at refrigeration temperatures over long periods of time (5, 14), one of the temperatures selected was 4°C. Excellent inhibition of *L. monocytogenes* occurred at both 4 and 32°C at pH 5.5 to 7.0 in APT broth. This inhibition lasted for at least 3 weeks at both temperatures, although some loss of viability was seen within 1 week in the controls at 32°C. It should be noted that these data resulted from an initial inoculum of 1.2×10^2 CFU/ml. With higher inocula, inhibition did occur but was generally less effective (data not shown). These results were obtained in a broth system, which allows uniform distribution of bacteriocin and is relatively free of interfering factors such as high fats, high protein, and high solids that are found in various food systems. Data obtained from broth systems often cannot be extrapolated to food systems; therefore, the effectiveness of PA-1 bacteriocin was investigated first in a more idealized broth system and then in several food systems.

Studies involving food systems showed that in mildly acidic and neutral environments *L. monocytogenes* grows well at refrigeration temperatures, confirming the psychrotrophic nature of the pathogen. In a relatively acidic system, exemplified by the creamed cottage cheese (pH 5.1), viable counts of the pathogen declined over 7 days at 4°C. Similar trends were noted by Ryser et al. (14) in creamed and uncreamed cottage cheese. Irvin (9) found a decline in the counts of *L. monocytogenes* below pH 5.5 in grass silage. The decline in counts was attributed to the acidic environment. However, our data demonstrate an immediate (24-h) bactericidal effect on *L. monocytogenes* (Table 2) above and beyond any cell death caused by acid in the system.

When bacteriocin PA-1 was added to food systems containing *L. monocytogenes*, there was a rapid decrease in viable numbers of the pathogen. In nonacidic foods, the numbers of viable *L. monocytogenes* increased within a week. Such a recovery was not observed in the cottage cheese system even in the sample containing 1/10 of the concentration of bacteriocin added to the nonacidic food systems. The difference in the recovery of *L. monocytogenes* in the acidic and nonacidic systems containing bacteriocin PA-1 reflects the synergistic effect of lactic acid and the bacteriocin on the pathogen.

Although there was a resurgence of the pathogen in neutral food systems within 7 days, the addition of the crude bacteriocin at higher concentrations or purified bacteriocin at low or moderate concentrations should confer a protective effect against the pathogen over a longer period. Additional work is necessary to confirm this before commercial use in food systems. The effectiveness of bacteriocin PA-1 in inhibiting *L. monocytogenes* in high-protein, high-fat food systems was clearly demonstrated in these studies. The results indicate that the bacteriocin is not bound by protein or fat. The effectiveness of bacteriocin PA-1 in controlling *L. monocytogenes* in acidic foods is indicated by our data.

Inhibition was evident over wide temperature and pH ranges and was particularly effective at low initial inocula of the pathogen, similar to those which could occur in various food systems. Bacteriocins can offer a safe and natural means of controlling undesirable bacterial contamination, particularly human pathogens such as *L. monocytogenes*. Bacteriocin PA-1 would be especially appropriate for such an application because *P. acidilactici* has been traditionally used as a starter in many food fermentations (13) and, hence, is considered generally regarded as safe for addition to foods.

At least two other plasmid-borne bacteriocins produced by *Pediococcus* spp. have been described (3, 7) which differ in plasmid size from the PA-1 plasmid in PAC 1.0. We do not know at present whether these bacteriocins are related at the genetic level. Although the work described here clearly demonstrates inhibition of *L. monocytogenes* by the PA-1 bacteriocin, further work is needed to purify PA-1 to homogeneity and to determine its mode of action, efficacy, and economics of application in food systems.

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