J. Microbiol. Biotechnol. (2001), **11**(5), 831–837

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Synergistic Effect of Citric Acid and Pediocin K1, a Bacteriocin Produced by *Pediococcus* sp. K1, on Inhibition of *Listeria monocytogenes*

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Received: May 2, 2001
Accepted: July 12, 2001

Abstract Pediocin K1, a bacteriocin produced by *Pediococcus* sp. K1 isolated from Korean traditional fermented flatfish, inhibited certain strains of *Lactobacillus, Streptococcus*, and *Listeria monocytogenes*. Pediocin K1 was found to be stable at 90°C for 30 min. Among the organisms tested, *Listeria monocytogenes* was the most sensitive to pediocin K1 and was completely killed when the initial inoculum size of *L. monocytogenes* cells was equal to or less than 10^3 CFU/ml. The degree of inhibition of *Listeria monocytogenes* by pediocin K1 increased 10-fold on the addition of citric acid (0.2%) to the medium, thereby showing the synergistic effect of citric acid. *Listeria monocytogenes* cells resistant to pediocin K1 appeared at a frequency of about 10^{-4}/cells. Once developed after exposure to pediocin K1, the resistant phenotype still persisted in the absence of pediocin K1 in successive cultures. This infers that resistance may be attributable to genetic change(s) in the resistant cells.

Key words: Pediocin K1, bacteriocin, *Listeria monocytoenes*

Many lactic acid bacteria (LAB) produce bacteriocins that supposedly confer an advantage on the producers' competing for food and available niches [15]. Over the last few decades, extensive efforts have been made to find novel LAB bacteriocins with desirable properties, such as broad inhibition spectra, and pH and heat stability [1, 7, 12]. Since many fermented foods containing LAB have been consumed for thousands of years, bacteriocins from LAB or LAB-producing bacteriocins are generally regarded as safe for human consumption. Therefore, bacteriocins from LAB are currently prime candidates as safe food-preservatives for replacing chemical preservatives, the safety of which has often been questioned [9]. Furthermore, bacteriocin-producing LAB have advantages over nonproducers, since bacteriocins have been shown to enhance the ability of probiotic strains to suppress the growth of undesirable bacteria in the human GI (gastrointestinal) tracts [5, 13]. Various functional foods containing probiotic strains are currently on the market worldwide [16]. LAB with strong bacteriocin activities against food poisoning bacteria such as *Listeria monocytogenes* or *Staphylococcus aureus* are very useful as probiotic strains. Various Korean fermented foods have been previously screened for novel bacteriocin producers. A *Lactococcus lactis* subsp. *lactis* strain was isolated from Kimchi and the bacteriocin inhibited *Listeria monocytogenes* and *Staphylococcus aureus* [11]. A *Lactococcus lactis* strain inhibiting *Lactobacillus delbrueckii* was also isolated from Jeot-gal [10]. A *Pediococcus* strain, *Pediococcus* sp. K1, has been isolated from fermented flatfish [8]. This paper reports on the inhibitory characteristics of the bacteriocin, pediocin K1, derived from *Pediococcus* sp. K1, against *Listeria monocytogenes*.

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Materials and Methods

Bacterial Strains and Culture Conditions

Pediococcus sp. K1 was isolated from traditional Korean fermented flatfish and the bacteriocin produced by *Pediococcus* sp. K1 was named pediocin K1 [8]. The indicator strains used in this study are listed in Table 1 and many were derived from humans. Human fecal samples were diluted and plated on selection media, each specifically designed for the recovery of organisms of different genera [6]. LAB and bifidobacteria were grown in a MRS broth (Difco Lab., Detroit, U.S.A.). The *Escherichia coli* and *Staphylococcus*

Table 1. Inhibition of indicator organisms by pediocin K1.

Indicator strains	S	P
Lactobacillus curvatus, KCA17012	++	++
Lactobacillus acidophilus, VPI11088	–	–
Lactobacillus delbrueckii, ATCC4797	–	–
Streptococcus thermophilus, th-116	–	–
Streptococcus sp. 1, 2	–	+
Streptococcus sp. 3, 7, 8	–	–
Staphylococcus sp. 5, 10, 12	–	–
Staphylococcus sp. 11	–	+
Staphylococcus aureus, ATCC25923	–	+
E. coli sp. 1-2	–	–
Listeria monocytogenes, ATCC1911	+++	+++
Clostridium perfringens sp. 1-6	–	–
Bifidobacterium RD-3, 64, BB-13, BGN-4	–	–

S: Supernatant containing pediocin K1.
P: Partially purified pediocin K1.

species were cultured in a brain heart infusion (BHI) broth. The *Listeria* species were grown in APT media, and the *Clostridium perfringens* in DS media.

Bacteriocin Detection

The bacteriocin activity was determined using the spot-on-lawn method [3]. One arbitrary unit (AU) of pediocin K1 was defined as the reciprocal of the greatest dilution of the bacteriocin that produced a clear inhibition zone.

Crude Pediocin K1 Preparation

Crude pediocin K1 samples were obtained by dissolving an ammonium sulfate pellet in sterile water. Ammonium sulfate powder was added to a 20 ml culture of *Pediococcus* sp. K1 at a final concentration of 90% (w/v). An ammonium sulfate pellet was recovered by centrifugation at 13,000 ×g for 1 h, then the resulting pellet was dissolved in 1 ml of sterile water. The pediocin K1 sample prepared in this way was designated P in Table 1. The supernatant of an overnight culture of *Pediococcus* sp. K1 was occasionally used as a bacteriocin sample. The pH of the overnight culture was adjusted to 6.5 with 3 N NaOH, and the culture was centrifuged at 4,000 ×g for 10 min. The supernatant was then transferred to a clean bottle and filter-sterilized using a 0.45 μm membrane filter. The pediocin K1 sample prepared in this way was designated S in Table 1. Unless otherwise indicated, prep P was used as the pediocin K1 sample in the experiments.

Sensitivity of Pediocin K1 to Heat and Proteinase K

A crude pediocin K1 prep (P) was heat-treated at 90°C for 30 min and then tested for its activity against *L. monocytogenes*. The pediocin K1 prep was also treated with proteinase K to demonstrate its proteinaceous nature. *L. monocytogenes* was used to inoculate (1%) 1 ml of APT media with 20% (v/v) supernatant containing pediocin K1 (S), with 50 μl of proteinase K (20 mg/ml in milliQ water) and added to one of two tubes. Both tubes were incubated at 37°C for 24 h with the optical densities (O.D.) of the cultures (610 nm) measured every hour.

Synergistic Effect of Citric Acid on the Inhibition of Indicators with Pediocin K1

Citric acid was added to the cultures, in either the presence or absence of pediocin K1 (800 AU/ml), to determine whether its addition had any synergistic antimicrobial effects. The concentrations of citric acid used were 0, 0.05, 0.1, and 0.2% (v/v) to a BHI broth inoculated with indicators (1%). Pediocin K1 was subsequently added to the culture, then the growth characteristics and viable cell counts of the indicators were measured at various intervals at 37°C.

Appearance of Pediocin K1-resistant *L. monocytogenes* Cells

Several single colonies of *L. monocytogenes* were picked from a BHI agar plate and used to inoculate the BHI broth separately. After incubation at 37°C for 24 h, aliquots of the cultures were spread on the BHI agar plates containing pediocin K1 (800 AU/ml) and on plates without pediocin K1. After incubation overnight at 37°C, the number of colonies on both plates was counted and the fractions of pediocin K1-resistant cells were calculated.

Effect of Preincubation Conditions on the Development of Resistance Against Pediocin K1

L. monocytogenes cells were cultivated under different conditions to examine whether the presence or absence of pediocin K1 in the media affected the development of resistance to the bacteriocin. Fresh APT broth was inoculated with *L. monocytogenes* (1%, v/v) for a maximum of 13 days at daily intervals (Table 2). The '+' sign in Table 2 indicates the presence of pediocin K1 (800 AU/ml) in the

Table 2. Cultivation schemes for *L. monocytogenes* in the presence or absence of pediocin K1 (800 AU/ml) to assess the effect the of cultivation conditions on development of pediocin K1 resistance.

	Times of transfer													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
I	–	+	+	+	–	–	–	+	+	+	–	–	–	±
II				–	+	+	+	–	–	–	+	+	+	±
III							–	+	+	+	–	–	–	±
IV										-	+	+	+	±
V												–	+	±
VI													–	±

+: Cultivation in medium with pediocin K1.
–: Cultivation in medium without pediocin K1.
±: Aliquots of culture on 14th day were spread onto plates containing pediocin K1 and plates without pediocin K1.

medium and the '–' sign indicates the absence of pediocin K1. After preincubation according to the schemes depicted in Table 2, fresh APT broth containing either pediocin K1 (800 AU/ml) or no bacteriocin was inoculated with preincubated *L. monocytogenes* cells. While incubating the samples for 30 h at 37°C, the appearance of resistant cells was noted and comparisons were made between the samples.

RESULTS AND DISCUSSION

Production and Antimicrobial Effect of Pediocin K1

Both the growth characteristics of *Pediococcus* sp. K1 in the MRS and the production of pediocin K1 during growth were examined (Fig. 1). As depicted in Fig. 1, the production of pediocin K1 started in the middle of the exponential growth phase, reaching to the maximum levels (6,500 AU/ml) in the stationary phase (around 27 h). The pH of the medium dropped from 6.17 to 4.04 during the 30 h incubation period. Pediocin K1 was found to inhibit both *Lactobacillus curvatus* and *Listeria monocytogenes* (Table 1). In addition, pediocin K1 prep P (see description under Materials and Methods) inhibited *Streptococcus* sp. 1, 2, and *Staphylococcus* sp. 11 (human origin), however, the supernatant (prep S) did not. As with the bifidobacteria strains, both the *Streptococcus* and *Staphylococcus* species with strain numbers used in the current study were derived from the human GI (gastrointestinal) tract. The difference between the inhibition patterns of preparations P and S in Table 1 might have been due to different pediocin K1 concentrations. This was because pediocin K1 prep P was concentrated using ammonium sulfate precipitation, whereas the supernatant (prep S) was not. Pediocin K1 did not inhibit *E. coli* or any of the other Gram-positive bacteria tested. Of the indicators tested, *L. monocytogenes* was found to be the most sensitive to pediocin K1. The growth

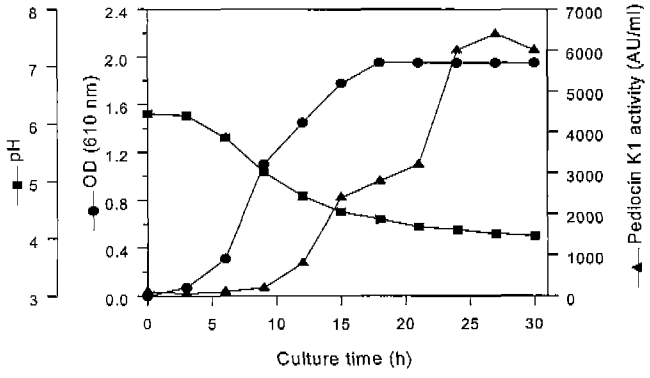

Fig. 1. Production of pediocin K1 by *Pediococcus* sp. K1 in MRS broth.
Symbols: optical density at 610 nm (-●-); pediocin K1 activity assayed against *L. monocytogenes* (-▲-); pH of the culture (-■-).

of *L. monocytogenes* cells in the APT broth containing 20% (v/v) pediocin K1 prep (S) was completely inhibited during the first 10 h of incubation. The O.D. (610 nm) values for the control and the pediocin K1-treated cultures were 0.71 and 0.03, respectively, after 10 h. However, the O.D. value for the culture including both proteinase K and pediocin K1 was 0.2, indicating the proteinaceous nature of pediocin K1.

Stability of Pediocin K1

Pediocin K1 was found to be resistant to heat treatment at 90°C for 30 min. This result indicated that pediocin K1 was heat-stable; a prerequisite for bacteriocins intended for use as food preservatives. Many bacteriocins belonging to either group I (lanthibiotics) or group II (small peptides) are heat stable and retain some activity even after autoclaving

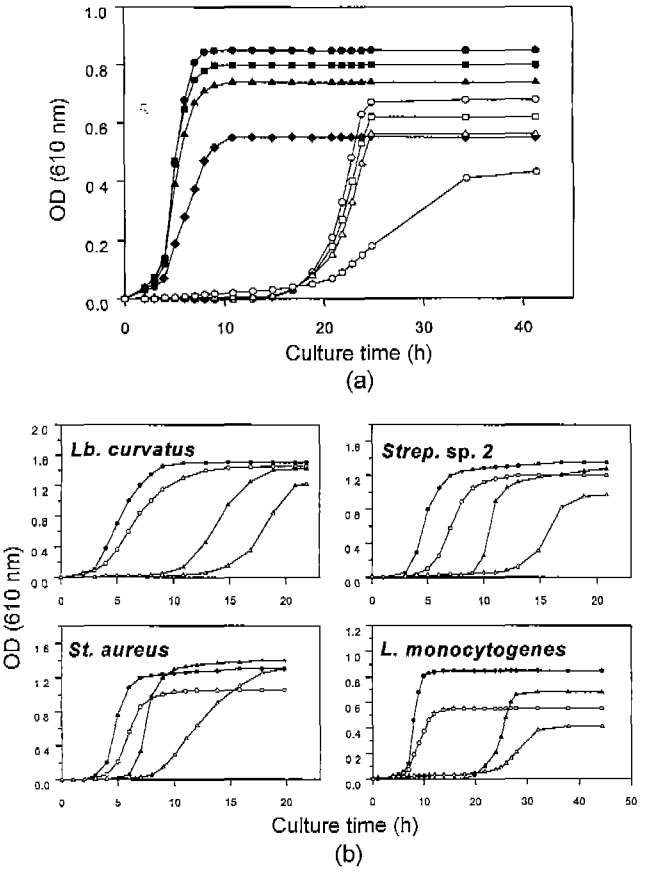

Fig. 2. Synergistic effect of citric acid on inhibition of indicators by pediocin K1.
(a) *L. monocytogenes* cells were inhibited by citric acid (0.05–0.2%) in the presence or absence of pediocin K1 (800 AU/ml). -●- control, -■- citric acid 0.05%, -▲- citric acid 0.10%, -◆- citric acid 0.20%, -○- pediocin K1, -□- citric acid 0.05% + pediocin K1, -△- citric acid 0.10% + pediocin K1, -◇- citric acid 0.20% + pediocin K1. (b) Various Gram-positive organisms were inhibited by citric acid (0.2%) in the presence or absence of pediocin K1 (800 AU/ml). -●- control, -○- citric acid, -▲- pediocin K1, -△- citric acid + pediocin K1.

(121°C, 15 min). Therefore, it is likely that pediocin K1 is either a class I or class II bacteriocin.

Synergistic Antimicrobial Effect of Pediocin K1 and Citric Acid

Citric acid was added to the cultures at a maximum concentration of 0.2% (v/v) to determine whether the inclusion of citric acid enhanced the antimicrobial activity of pediocin K1 on the indicators in a synergistic manner. Citric acid alone inhibited the growth of *L. monocytogenes*, with the degree of inhibition increasing as a function of the citric acid concentration (Fig. 2). As shown in Fig. 2, at a concentration of 0.2%, citric acid significantly inhibited *L. monocytogenes* (Fig. 2a) and the other Gram-positive bacteria tested (Fig. 2b). When citric acid and pediocin K1 (800 AU/ml) were added to the culture simultaneously, all the indicators tested were inhibited to an extent greater than with just citric acid or pediocin K1 alone. The effect of the simultaneous addition of pediocin K1 and citric acid on all four of the indictors tested exhibited an extension of the lag phase and a reduction in the maximum O.D. value. For *L. monocytogenes*, the decrease in the maximum O.D. values showed a linear reduction in the number of viable cell numbers (Fig. 3). During the 30-h cultivation period, a 2 log scale difference in the number of viable cells was observed between cultures exposed to 0.2% citric acid alone and cultures treated with citric acid and pediocin K1 simultaneously. These findings clearly showed a synergistic antimicrobial effect of citric acid. Nykänen *et al.* [14] reported that the combination of lactic acid and nisin whey permeate was more effective against several Gram-positive organisms than single treatments of either lactic acid or nisin whey permeate alone. More importantly, the combined treatment inhibited the growth of some Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, more efficiently than lactic acid alone. Generally, Gram-negative bacteria are insensitive to bacteriocins from Gram-positive bacteria, unless cell walls were damaged by stress or EDTA treatment. Therefore, the combined use of pediocin K1 and citric acid may improve the inhibitory spectrum of pediocin K1, as suggested by other researchers [2, 4].

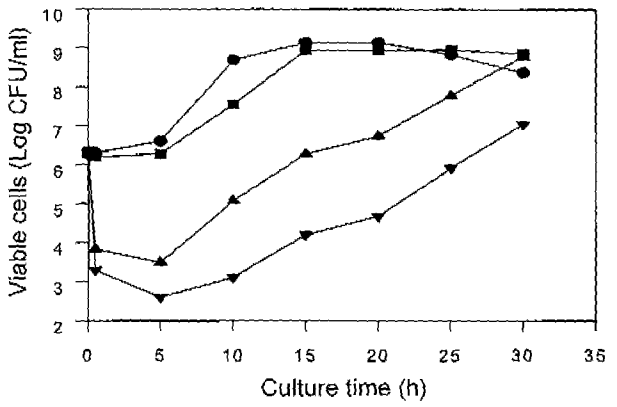

Fig. 3. Viable counts of *L. monocytogenes* during growth in MRS broth in the presence of citric acid (0.2%) and/or pediocin K1 (800 AU/ml).
-●- control, -■- citric acid, -▲- pediocin K1, -▼- citric acid + pediocin K1.

Viability of Indicator Relative to Inoculum Size

The viability of *L. monocytogenes* cells in the co-presence of pediocin K1 (800 AU/ml) and citric acid (0.2%) was examined using varying amounts of inocula. The growth of *L. monocytogenes* was not detected for up to a 7-day period, during which the size of the inoculum was equal to or less than 10^4 CFU/ml (Fig. 4a). Once the inoculum size was increased to 10^7 CFU/ml, growth started the following day, yet the maximum O.D. values (610 nm) were only 1/3

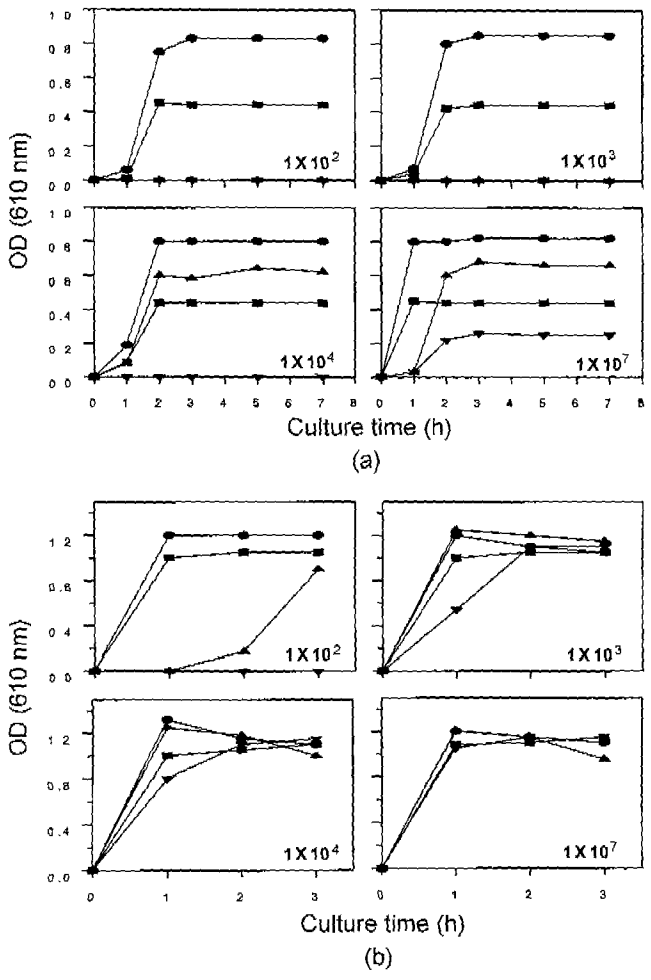

Fig. 4. Growth of indicators in presence of citric acid (0.2%) and/or pediocin K1 (800 AU/ml) with different inoculum sizes.
The inoculum sizes in CFU/ml units are shown in the figure. (a) *L. monocytogenes*. (b) *Streptococcus* sp. 2 (human isolate). -●- control, -■- citric acid, -▲- pediocin K1, -▼- citric acid + pediocin K1.

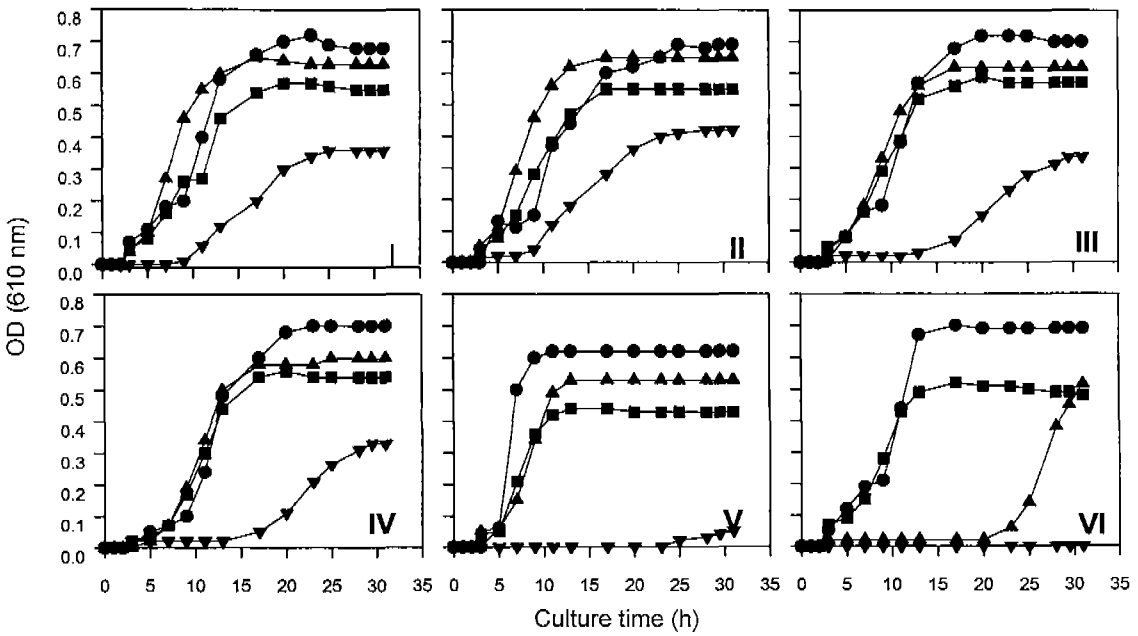

Fig. 5. Growth of *L. monocytogenes* cells in the presence of citric acid (0.2%) and/or pediocin K1 (800 AU/ml).
L. monocytogenes cells (I to VI) were cultivated previously under different conditions (see Table 2 for the descriptions). -●- control, -■- citric acid, -▲- pediocin K1, -▼- citric acid + pediocin K1.

that of the control culture (cells with no inhibitors). Figure 4 also indicates that pediocin K1 applied during isolation completely killed *L. monocytogenes* cells with the initial inoculum size of 10^3 CFU/ml or less. When the initial inoculum size was 10^3 CFU/ml, pediocin K1-resistant cells appeared rapidly when the O.D. (610 nm) value reached about three quarters of the control culture. These results were in support of the observation that pediocin K1-resistant *L. monocytogenes* cells appeared at a frequency of 10^{-4}/cell (see below). In the presence of 0.2% citric acid, pediocin K1 killed 10 times as many *L. monocytogenes* cells. Citric acid alone killed about half of the indicator cells, regardless of the inoculum size. The viability of *Streptococcus* sp. 2 cells in the presence of pediocin K1 was much higher than that of *L. monocytogenes*, (about 100 times, see Fig. 4b), again indicating the increased sensitivity of *L. monocytogenes* to pediocin K1. Combined-treatment of citric acid (0.2%) and pediocin K1 inhibited the growth of *Streptococcus* sp. 2 when the inoculum size was less than or equal to 10^2 CFU/ml. However, when the inoculum size was larger than 10^2 CFU/ml, the combined-treatment of pediocin K1 and 0.2% citric acid had no effect on the growth of *Streptococcus* sp. 2.

Appearance of Pediocin K1-Resistant Indicator Cells and Some Observations on Their Resistance

L. monocytogenes cells resistant to pediocin K1 appeared at a constant frequency, estimated at 10^{-4}/cell. When *L. monocytogenes* cells were cultivated in an APT broth under the different conditions described in Table 2, resistant cells appeared rapidly and the number of resistant cells in the APT broth with pediocin K1 (800 AU/ml) was almost the same as the number of cells in the APT broth without pediocin K1, as inferred by the O.D. (610 nm) values (Fig. 5). The length of exposure to pediocin K1 was apparently not important in the development of resistance. As shown in Fig. 5, even a single day of exposure (sample V) was enough to induce a significant degree of resistance. However, the number of viable cells under the co-presence of pediocin K1 and citric acid (0.2%) was much lower in the cultures exposed for a day (sample V) than in the cultures exposed for longer (minimum of 3 days for other samples in Table 2). Extended exposure to pediocin K1 did not increase the level of resistance, provided that a certain minimum period of exposure had occurred. For example, the growth patterns of resistant cells in sample I (+++/---, 2 cycles) and III (+++/---, 1 cycle) were almost identical. The order of pediocin K1 inclusion/omission to the culture was not important in the development of resistance. In sample 3, *L. monocytogenes* cells were cultivated in the presence of pediocin K1 for three consecutive days followed by cultivation for three days in the absence of pediocin K1 (+++/---). Similarly, in sample IV (and sample II, too), the indicators were exposed to pediocin K1 for three days immediately before plating. However, in both cases, the patterns of resistant cell growth were the same. This observation indicates that once resistance is attained through exposure to pediocin K1, the phenotype is maintained regardless of the presence or absence of pediocin K1 in the medium. Given that resistance was

maintained even when cells were cultivated in the absence of pediocin K1, it would appear that resistance can be attributed to genetic change(s) in *L. monocytogenes*. Davies *et al.* [4] reported that nisin resistance in *L. monocytogenes* was related to a change in the cell wall properties, and the hydrophobicity of the cell wall of the nisin-resistant mutants was less than that of the wild-type strain. Crandall and Montville [2] also attributed nisin resistance in *L. monocytogenes* to alterations in the cytoplasmic membrane and the cell wall. Unlike changes in the fatty acid composition, cell wall alterations are constitutive, thereby implying that certain genetic alteration(s) may be responsible. Obviously, further studies on the nature of pediocin K1 resistance are required. An interesting observation was made when *L. monocytogenes* cells were cultivated in the presence of pediocin K1 (800 AU/ml) and the activity of pediocin K1 in the medium was measured during cultivation. The activity of pediocin K1 in the culture of resistant cells remained higher than that in the culture of sensitive cells (cells not exposed previously to pediocin K1). As shown in Fig. 6, the pediocin K1 activity in the resistant cell culture was 500 AU/ml after 30 h; 2.5-fold higher than that in the sensitive cells. This might be due to the absorption of pediocin K1 by receptors on the surface of the sensitive cells.

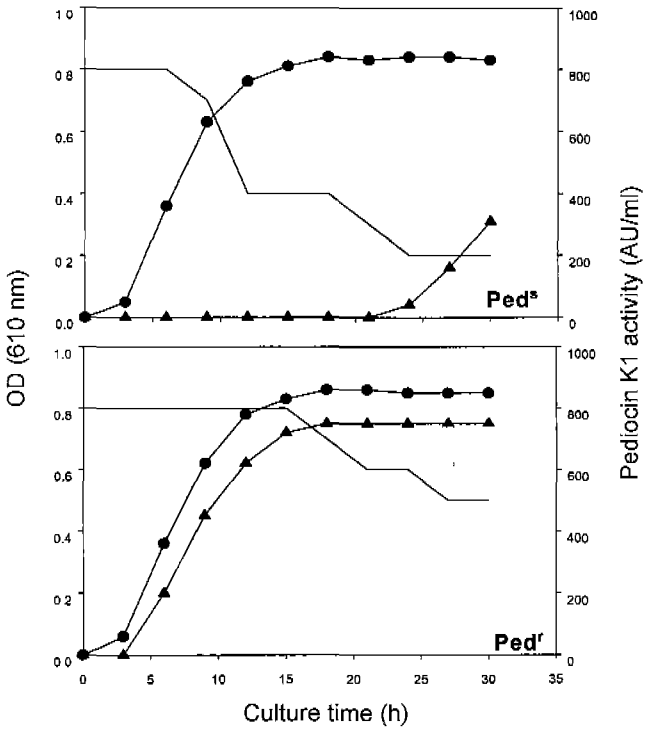

Fig. 6. Change in pediocin K1 activity during cultivation of *L. monocytogenes* in the presence of pediocin K1.
-●-: cultivation of *L. monocytogenes* cells in medium without pediocin K1. -▲-: cultivation of *L. monocytogenes* cells in medium with pediocin K1 (800 AU/ml). ——: pediocin K1 activity during cultivation of *L. monocytogenes* cells. Ped^s: *L. monocytogenes* strain (wild-type) sensitive to pediocin K1. Ped^r: *L. monocytogenes* strain resistant to pediocin K1.

Acknowledgments

This work was supported by research grants from the Korean Ministry of Health and Welfare (HMP-98-F-5-0020) and Brain Korea 21. The authors are grateful for the financial support.

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