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Antibiosis of Pediocin-Producing *Pediococcus* sp. KCA1303-10 Against *Listeria monocytogenes* in Mixed Cultures

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Abstract Pediocin K1 is a bacteriocin produced by *Pediococcus* sp. KCA1303-10, isolated from traditionally fermented flatfish in Korea. Pediocin K1-dependent antibiosis and pediocin K1-independent antibiosis against *Listeria monocytogenes* were investigated by comparing antibiosis potential of the ped+ wild-type strain of *Pediococcus* sp. KCA1303-10 with that of the ped- mutant strain in 3 different media at 3 different temperatures. In the synthetic MRS-APT medium, bacteriocin (pediocin K1)-dependent antibiosis (BDA) acted as the major driving force of overall antibiosis at the initial stage before the pH of the media was not sufficiently lowered, while bacteriocin-independent antibiosis (BIA) took over the major role at the late stage of antibiosis by killing otherwise resistant cells in the medium. The role of BDA increased as the temperature of the system decreased. The antibiosis potential of BDA among the overall antibiosis of *Pediococcus* against *Listeria* at 37°C was calculated as 46%, and as 75% at 25°C. In the skim milk medium, antibiosis of *Pediococcus* against *Listeria* was weakened more than 4 log cycles compared to that of the synthetic medium; however, BDA worked as the main antibiosis force regardless of the culturing temperature in the skim milk medium. In the bean soup medium, BDA also worked as the major killing mechanism against *Listeria*, but BIA played as another suppressing mechanism against otherwise pediocin-resistant *Listeria* population. These results suggest that a large portion of the inhibitory action of the ped+ *Pediococcus* sp. KCA1303-10 was attributable to the bacteriocin produced by the strain and that viable *Pediococcus* sp. KCA1303-10 was superior to the purified bacteriocin in suppressing the occurrence of the bacteriocin-resistant *Listeria monocytogenes* in food systems.

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Bacteriocins are antimicrobial polypeptides produced by bacteria with which they attack other microorganisms in the ecosystem, resulting in better survival and domination of their producers over the microbial ecosystems such as fermented foods or human digestive tracts [2, 15]. Among a variety of bacteriocin producers, lactic acid bacteria (LAB) have attracted more intensive interest from industry, owing to their potential of development into a valuable source of biopreservative to prevent food-borne pathogens, such as *Listeria monocytogenes*, from growing in the food system [11, 16].

Pediococcus sp. KCA1303-10 was isolated from fermented flatfish [9]. It produced a bacteriocin, pediocin K1, which showed strong inhibitory activitiy against *L. monocytogenes*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Lactobacillus curvatus* [9]. Pediocin K1 is a heat-stable bacteriocin of 4,200 dalton molecular mass with amino terminal sequences similar to pediocin PA-1. Genes responsible for the production of the pediocin K1 and immunity to it are encoded by the 9.1-kb plasmid (pCA9.1). On exposure to the pediocin K-1, viable cell numbers of *L. monocytogenes* are immediately reduced by 4 log cycles, but they tend to acquire some resistance on an extended exposure [10]. This occurrence of bacteriocin-resistant *Listeria* has been a major hurdle in using bacteriocins and/or bacteriocin-producing LAB in food systems [6]. Recently, it was reported that the resistance was due to the molecular change in the mannose-specific phosphotransferase system EII^{Man} in the membrane of *Listeria* which functions normally as the docking molecule for the class IIa bacteriocin such

as pediocin [6, 7, 14]. In the previous report, it was shown that citric acid had a good synergistic effect with cell-free, purified pediocin K1 in suppressing the appearance of bacteriocin-resistant *Listeria* [10]. The role played by the citric acid in the simulated system may be played by the lowered pH, organic acids such as lactic acid, and hydrogen peroxide in the real biological system [3, 12, 13], which can be collectively described as bacteriocin-independent antibiosis (BIA) in bacteriocin-producing lactic acid bacteria. Furthermore, our preliminary experiments showed that using bacteriocin-producing *Pediococcus* was far more efficient in antibiosis against *Listeria* than cell-free bacteriocin. In this context, the role of bacteriocin-dependent antibiosis (BDA) and bacteriocin-independent antibiosis (BIA) against *L. monocytogenes* was investigated in the present study by comparing antibiosis potential of the ped+ wild-type strain of *Pediococcus* sp. KCA1303-10 with that of ped- mutant strain in 3 different media systems at 3 different temperatures.

Materials and Methods

Bacterial Strains and Culturing Conditions

Pediococcus sp. KCA1303-10 was originally isolated from a traditionally fermented flatfish [9]. It produced pediocin K1 which was effective against *Listeria monocytogenes* ATCC1911. *Pediococcus* sp. KCA1303-10 has four residential plasmids, among which pCA9.1 was responsible for the production of the pediocin K1 and for the immunity to itself. *Pediococcus* sp. KCA1303-10C was the ped- mutant strain which was cured of the pCA9.1. *Pediococcus* strains were cultured in the MRS broth (Difco, Becton Dickson, France), and *L. monocytogenes* ATCC1911 in APT broth (Difco, Becton Dickson, France). Working cultures were subcultured twice in the appropriate broth medium and incubated at 37°C for 12 h before use.

Bacteriocin Detection and Activity Assay

For detection of antagonistic activity, the spot-on-lawn method was used [1, 9]. Seven ml of MRS soft agar (0.75%) containing 5×10^7 CFU of the indicator strain was poured over the 1.5% agar plate. Ten μl of cell-free supernatant of pediocin K1 producer strain was spotted onto the overlaid surface, and the plates were incubated for 24- 36 h at the growth temperature of the indicator strain and checked for formation of inhibition zones. The activity of bacteriocin was assayed by spotting two-fold serial dilutions of supernatant onto the indicator lawn. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution to show an inhibition zone.

Preparation of Partially Purified Pediocin K1

The pediocin-producing strain was grown to the stationary phase by incubating at 37°C for 18 h in 1 liter of MRS broth. Crude pediocin K1 was prepared as follows. The bacteria were removed by centrifugation (8,000 rpm for 15 min at 4°C) and the supernatant was passed through a 0.45 μm pore-size filter membrane. Ammonium sulfate was added to achieve 50% saturation. After precipitation for 24 h at 4°C, the precipitate was centrifuged at 8,000 rpm for 15 min at 4°C. The pellet was resuspended with 15 ml of distilled water and dialyzed against 1 liter of 10 mM phosphate buffer (pH 7.0) for 24 h at 4°C.

Calculation of Viable Cell Numbers of *L. monocytogenes*

During incubation, aliquots of the cultured broths were removed at specific time intervals. The aliquots were diluted 100-fold by distilled water. After 3 consecutive serial dilutions, 10 μl of each sample was spotted onto Oxford agar plates. The plates were incubated at 37°C for 24- 36 h. The black colonies were counted for viable cell numbers of *L. monocytogenes*. The same experiment was performed 3 times, and viable cell numbers were determined by the mean number.

Assessment of Antibiosis of *Pediococcus* sp. KCA1303-10 Against *L. monocytogenes* in Synthetic MRS-APT Medium

L. monocytogenes was co-cultured with *Pediococcus* sp. KCA 1303-10 or treated with crude pediocin K1 at 15°C, 25°C, and 37°C for 30 h in synthetic medium (MRS broth + APT broth). The synthetic medium was prepared by dissolving 55 g MRS and 47.5 g APT into 1 liter of distilled water and autoclaved at 121°C for 15 min. Overnight culture of *Pediococcus* sp. KCA 1303-10 was inoculated into the medium at 1% inoculum. When necessary, pediocin K1 was added into each medium at the concentration of 800 AU/ml. *L. monocytogenes* was inoculated into each medium at 10^6 CFU/ml. Viable cell numbers and pH of the medium were measured every 3 h during incubation for 30 h by the method described above. The same experiments were carried out at three different temperatures, 15°C, 25°C, and 37°C.

Assessment of Antibiosis of *Pediococcus* sp. KCA1303-10 Against *L. monocytogenes* in Skim Milk

The same experimental procedure as described above was employed for the assessment of antibiosis of *Pediococcus* sp. KCA1303-10 against *L. monocytogenes* in the skim milk medium. The skim milk medium was prepared by dissolving 100 g of skim milk powder (Difco, Becton Dickson, France) in 1 liter of distilled water and autoclaved at 121°C for 15 min.

Assessment of Antibiosis of *Pediococcus* sp. KCA1303-10 Against *L. monocytogenes* in Bean Soup

The same experimental procedure as described above was employed for the assessment of antibiosis of *Pediococcus* sp. KCA1303-10 against *L. monocytogenes* in the bean

soup medium. The bean soup medium was prepared by dissolving 20 g of soybean powder in 1 liter of distilled water and autoclaved at 121°C for 15 min.

Antibiosis Potential

The effect of bacteriocin-dependent antibiosis (BDA) and bacteriocin-independent antibiosis (BIA) was assessed by calculating the area occupied by the graph, demonstrating the changes of the viable cell numbers of *L. monocytogenes* after treatment by pediocin K1 or pediocin K1-producing strain, and expressed as antibiosis potential (h×logCFU/ml).

RESULTS AND DISCUSSION

Antibiosis of *Pediococcus* sp. KCA1303-10 Against *L. monocytogenes* in Synthetic MRS-APT Medium

Antibiosis effects of ped+ and ped- *Pediococcus* sp. KCA1303-10 against *L. monocytogenes* in synthetic MRS-APT broth medium at 15°C, 25°C, and 37°C are illustrated in Fig. 1, showing the changes in viable cell numbers of the *L. monocytogenes*. The effect of cell-free pediocin on the survival of *L. monocytogenes* was also monitored to compare with that of pediocin-producing *Pediococcus* sp. KCA1303-10. The change in pH of the medium during growth of *Pediococcus* is also demonstrated in Fig. 2. As shown in Fig. 1, addition of cell-free bacteriocin into the medium immediately reduced viable cell numbers of *L. monocytogenes* by 4 log cycles within the first 3 h. The killing mechanism within the first 3 h was bactericidal and independent of the culturing temperature. However, this listeria-cidal effect turned into a static one, as the pediocin-resistant survivors appeared after 3 h. These pediocin-resistant cells grew into a larger population, as the incubation temperature came close to their optimum temperature (Figs. 1B and 1C). The rate of pediocin-resistant cell appearance was approximately 10^{-4}, in agreement with the previous report [10]. The cause of the appearance of the bacteriocin-resistant cells might be attributed to the spontaneous mutation on the receptor system EII^{Man} in the membrane [6, 7, 14].

However, as shown in Fig. 1C, co-culturing *L. monocytogenes* with the pediocin-producing cells of *Pediococcus* sp. KCA1303-10 at 37°C completely killed *L. monocytogenes*, preventing pediocin-resistant cells from appearing in the culture. This bactericidal effect started to appear after 6 h of co-culturing and completed at 15 h. This effect coincided with the growth-dependent production of pediocin into the medium by the strain KCA1303-10 [8] as well as the drop of pH from pH 6.2 at 3 h to pH 4.7 at 15 h (Fig. 2C). Figure 1C shows that co-culuring non-bacteriocinogenic mutant strain KCA1303-10C with the *L. monocytogenes* also resulted in complete inhibition of the listerial growth at 24 h. The mutant strain did not produce pediocin at all, because of its loss of the plasmid pCA9.1

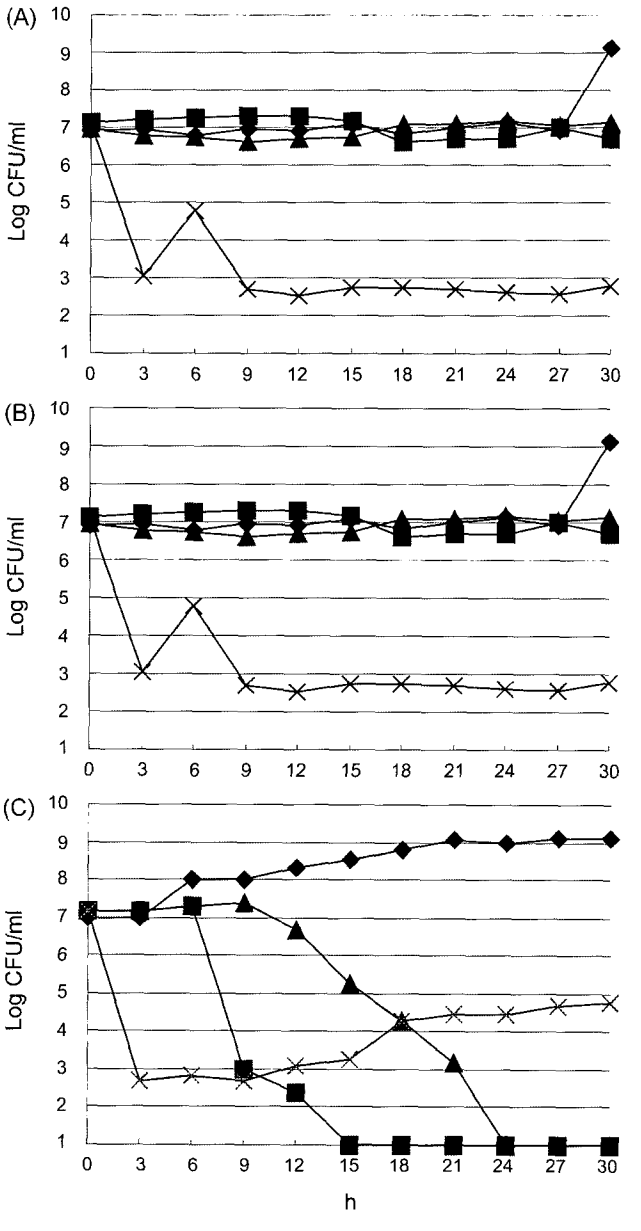

Fig. 1. Antibiosis of *Pediococcus* sp. KCA1303-10 on *Listeria monocytogenes* in synthetic MRS-APT medium.
Survived cells of *L. monocytogenes* are expressed as colony forming units (CFU)/ml. Experiment was performed at 15°C (A), 25°C (B), and 37°C (C). ◆, *L. monocytogenes* without any treatment. ■, *L. monocytogenes* co-cultured with ped+ wild-type strain *Pediococcus* sp. KCA1303-10. ▲, *L. monocytogenes* co-cultured with ped- mutant strain *Pediococcus* sp. KCA1303-10C. ×, *L. monocytogenes* treated with cell-free pediocin K1.

which was responsible for the bacteriocin production [9]. Therefore, its bactericidal effect seemed to be due to bacteriocin-independent antibiosis (BIA) mechanisms, including the drop of medium pH below 5.0, as illustrated in Fig. 2C.

Therefore, the antibiosis effect of co-culturing bacteriocinogenic *Pediococcus* with the *Listeria* seemed to

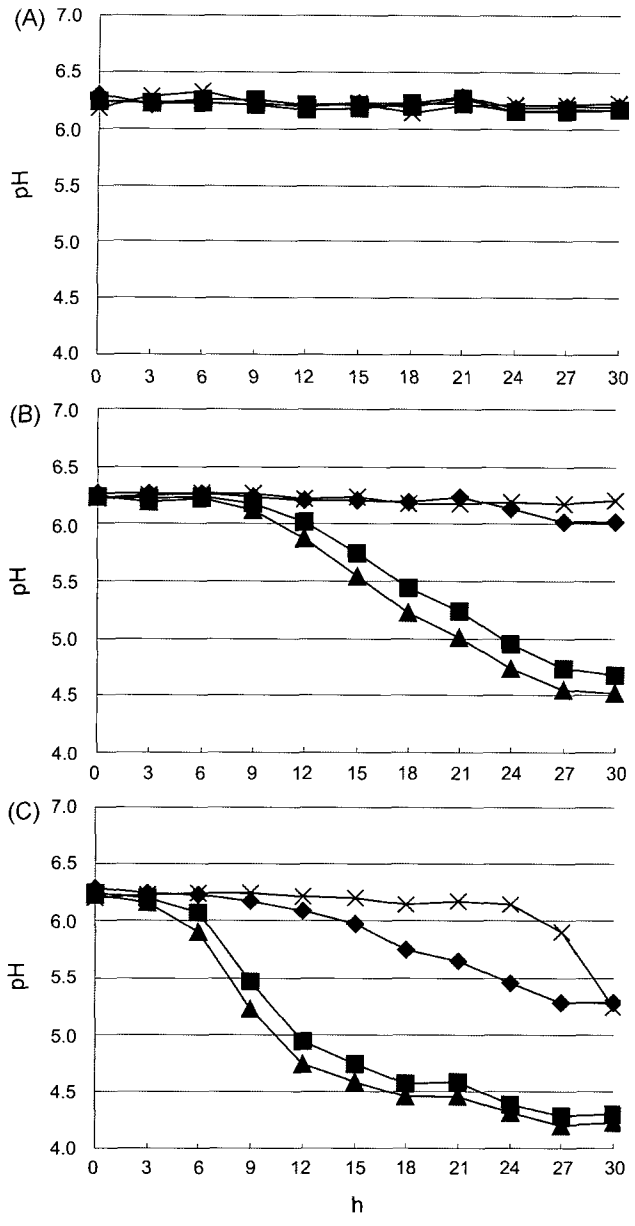

Fig. 2. Change of pH in culture medium during antibiosis of *Pediococcus* sp. KCA 1303-10 on *Listeria monocytogenes* in synthetic MRS-APT medium.
Experiment was carried out at 15°C (A), 25°C (B), and 37°C (C). ◆, *L. monocytogenes* without any treatment. ■, *L. monocytogenes* co-cultured with ped+ wild-type strain *Pediococcus* sp. KCA1303-10. ▲, *L. monocytogenes* co-cultured with ped- mutant strain *Pediococcus* sp. KCA1303-10C. ×, *L. monocytogenes* treated with cell-free pediocin K1.

be a two-fold mechanism: firstly, by direct killing of the listeria cell by the bacteriocin molecules, and secondly by the growth inhibition of the bacteriocin-resistant cells by the lowered pH. Another important benefit of co-culturing bacteriocinogenic *Pediococcus* was that the time required to remove listeria cells from the medium could be shortened by 9 h which was larger than the time required by the non-bacteriocinogenic strain (Fig. 1C). The effect of co-culturing was reduced when it was done at 25°C, as shown in Fig. 1B. The reduced effect seemed to be due to the reduced growth rate of the *Pediococcus* strain at the temperature as well as the decreased rate of pH lowering (Fig. 2B). There was no growth of the *Pediococcus* at 15°C. The faster lowering of media pH by the mutant strain than wild-type strain (Fig. 2) could be related to the absence of plasmid in the mutant cell, resulting in lessening extra energy burden to the mutant cell.

Contribution of Bacteriocin Production by *Pediococcus* sp. KCA1303-10 to Total Antibiosis Against *L. monocytogenes*

Comparison of the bactericidal patterns of bacteriocin-producing strain with its mutant which did not produce bacteriocin, will show how much BDA or BIA potential they have during their interaction against *L. monocytogenes*. Figure 3 summarizes their BDA and BIA potentials, which were calculated from Figs. 2B and 2C by integrating the graph by 3 h periods. As shown in Figs. 3A and 3B, BDA worked as the major driving force of the antibiosis at the beginning stage, when the pH of the media was not

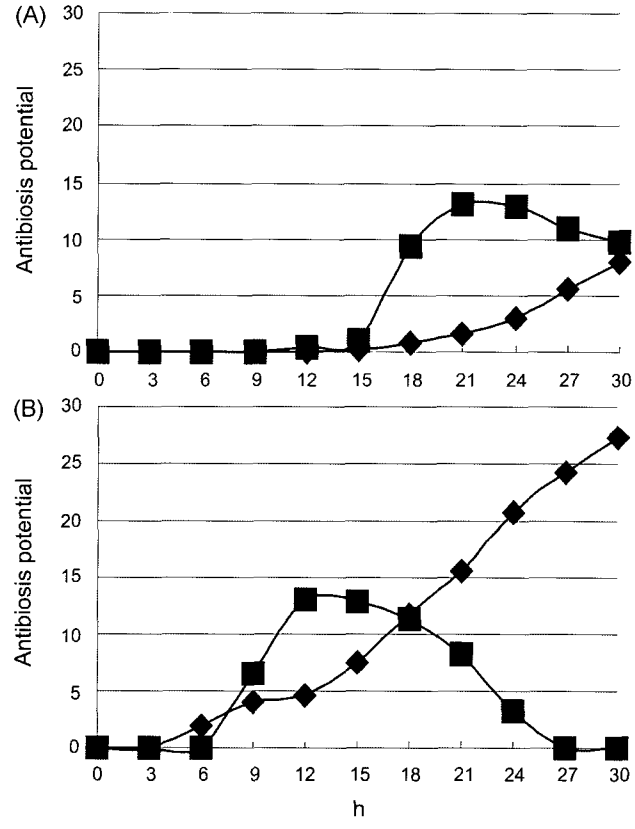

Fig. 3. Change of antibiosis potential of *Pediococcus* sp. KCA 1303-10 on *Listeria monocytogenes* in synthetic MRS-APT medium at 25°C (A) and 37°C (B).
Bacteriocin-dependent antibiosis potential is expressed as BDA (■), and bacteriocin-independent antibiosis potential as BIA (◆).

sufficiently lowered yet. However, BIA took over the major role at the later stage of antibiosis, killing otherwise resistant cells in the media. The role of BDA increased as the temperature of the system decreased (Fig. 3A). Based on accumulated integration of Fig. 3, the portion of BDA among the total antibiosis of *Pediococcus* at 37°C was calculated as 46% during a 3 h to 24 h period, while the portion of BDA at 25°C was calculated as 75% during a 9 h to 30 h period.

Antibiosis of *Pediococcus* sp. KCA1303-10 Against *L. monocytogenes* in Skim Milk

The inhibitory activity of *Pediococcus* sp. KCA1303-10 against *L. monocytogenes* was assessed in the skim milk,

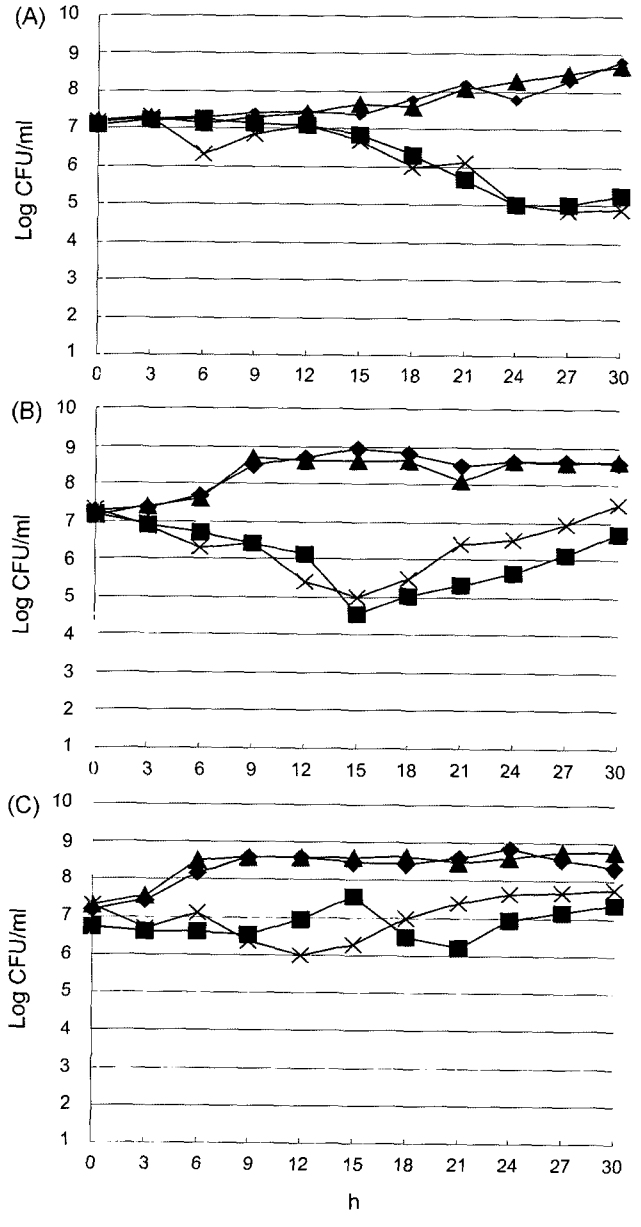

Fig. 4. Antibiosis of *Pediococcus* sp. KCA1303-10 on *Listeria monocytogenes* in skim milk medium. Survived cells of *L. monocytogenes* are expressed as colony forming units (CFU)/ml. Experiment was done at 15°C (A), 25°C (B), and 37°C (C). ◆, *L. monocytogenes* without any treatment. ■, *L. monocytogenes* co-cultured with ped+ wild-type strain *Pediococcus* sp. KCA1303-10. ▲, *L. monocytogenes* co-cultured with ped- mutant strain *Pediococcus* sp. KCA1303-10C. ×, *L. monocytogenes* treated with cell-free pediocin K1.

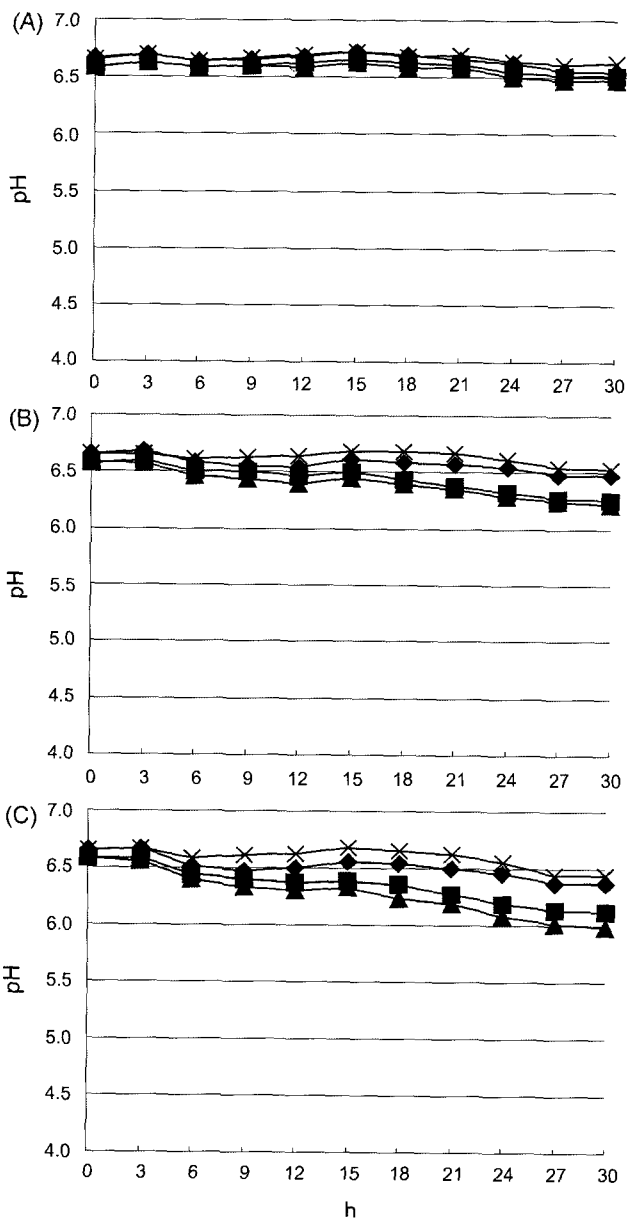

Fig. 5. Change of pH in culture medium during antibiosis of *Pediococcus* sp. KCA 1303-10 on *Listeria monocytogenes* in the skim milk.

Experiment was done at 15°C (A), 25°C (B), and 37°C (C). ◆, *L. monocytogenes* without any treatment. ■, *L. monocytogenes* co-cultured with ped+ wild-type strain *Pediococcus* sp. KCA1303-10. ▲, *L. monocytogenes* co-cultured with ped- mutant strain *Pediococcus* sp. KCA1303-10C. ×, *L. monocytogenes* treated with cell-free pediocin K1.

as demonstrated in Fig. 4 and Fig. 5. The results manifested 3 major differences in the interactions between bacteriocin, bacteria, and medium: first, *Listeria* grew better in skim milk than in synthetic MRS-APT media; second, there was almost no change of pH in skim milk even during bacterial growth; third, bacteriocin showed far reduced bacteriostatic activity against *Listeria* in skim milk. The first and second difference in the skim milk may explain why dairy products are apt to listerial infection during food processing or marketing. The buffering capacity of skim milk prevents the pH from dropping below 6.0, as shown in Fig. 5, resulting in better growth of *Listeria*. The third difference might result from absorption of bacteriocin to the components of the skim milk or partial inactivation by proteases in the skim milk [4]. The buffering activity of skim milk made a big change in the role of BDA and BIA potential in the system. As illustrated in Fig. 4, the mutant strain did not affect the growth of *Listeria* regardless of culturing temperature, as it could not change the pH of the media. Therefore, the antibiosis effect illustrated in Fig. 4, even if it was only a log 2 to 4 reduction in the listerial population, was considered mostly to be due to the BDA effect. These results suggest that bacteriocin or bacteriocin-producing lactic acid bacteria would be a highly useful biopreservative system in dairy foods, compared to other pH-lowering organic compounds or acids.

Antibiosis of *Pediococcus* sp. KCA1303-10 Against *L. monocytogenes* in Bean Soup

The effect of pediocin-producing *Pediococcus* sp. KCA1303-10 against *L. monocytogenes* in the bean soup was summarized in Fig. 6 and Fig. 7, and compared with that of cell-free pediocin or ped- strain *Pediococcus* sp. KCA1303-10. Figures 6 and 7 suggest that interactions of bacteriocin-bacteria-medium in the bean soup system were different in 3 ways from those in the previous two media. First, ped+ strain grew very well in the bean soup media while ped- mutant strain did not, as shown in Figs. 7B and 7C. Therefore, it was difficult to assess the contribution of BIA in the total antibiosis exerted by the ped+ strain in Fig. 6. However, comparison between inhibitory patterns of ped+ strain at 25°C (Fig. 6B) and at 37°C (Fig. 6C) led us to an apparent conclusion that the first 4 log cycle reduction within 3 h was achieved by BDA effect, and that antibiosis after 3 h was a net result from BIA action, including lowered pH of the media by the producer strain itself. Thirdly, the effect of cell-free pediocin in the bean soup medium was similar to that in the synthetic MRS-APT medium at 15°C, but different from it at 25°C and 37°C. It rapidly lost its anti-listerial effect after the first 3 h, as shown in Figs. 6B and 6C. The reason for unavailability of the bacteriocin in this experiment was not clear. It was noteworthy that ped+ strain could grow in the bean soup medium, whereas ped- strain could not, and the only difference between them was

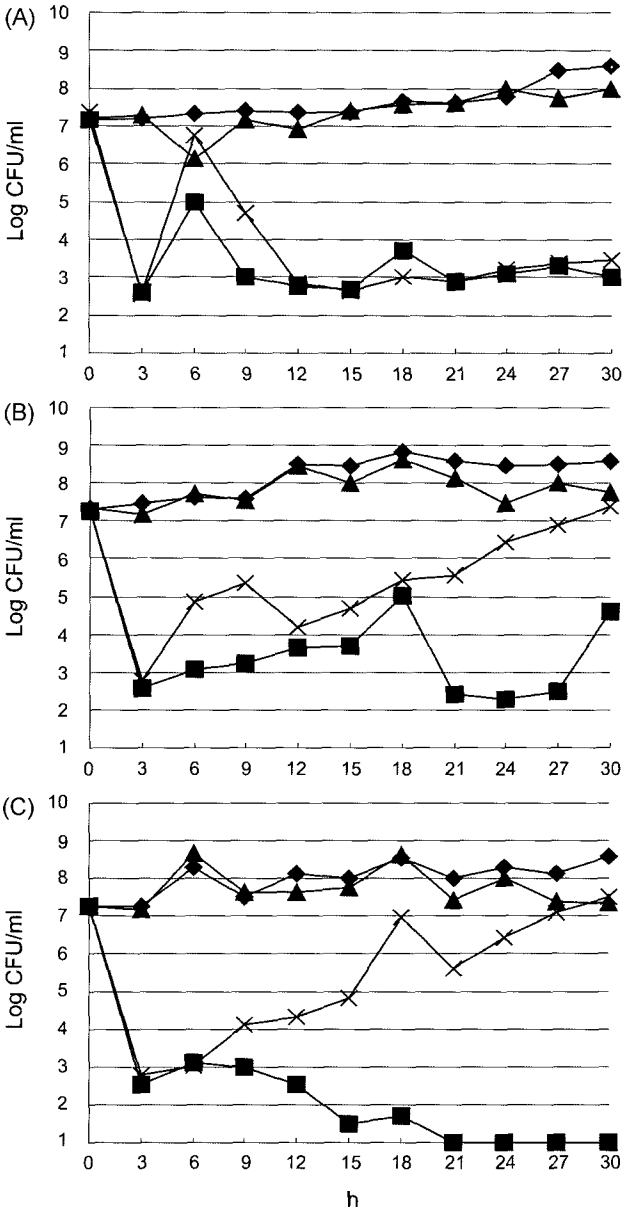

Fig. 6. Antibiosis of *Pediococcus* sp. KCA1303-10 on *Listeria monocytogenes* in bean soup medium.
Survived cells of *L. monocytogenes* are expressed as colony forming units (CFU)/ml. Experiment was done at 15°C (A), 25°C (B), and 37°C (C). ◆, *L. monocytogenes* without any treatment. ■, *L. monocytogenes* co-cultured with ped+ wild-type strain *Pediococcus* sp. KCA1303-10. ▲, *L. monocytogenes* co-cultured with ped- mutant strain *Pediococcus* sp. KCA1303-10C. ×, *L. monocytogenes* treated with cell-free pediocin K1.

the presence or absence of the residential plasmid pCA9.1. Plasmid pCA9.1 contains 13 open reading frames (ORF) (unpublished data). Among them, 7 ORFs are genes with known functions, including pedA, pedB, pedC, and pedD, which are responsible for the production of pediocin and immunity to the pediocin. There are 6 ORFs on the plasmid with unknown functions. Further elucidation of

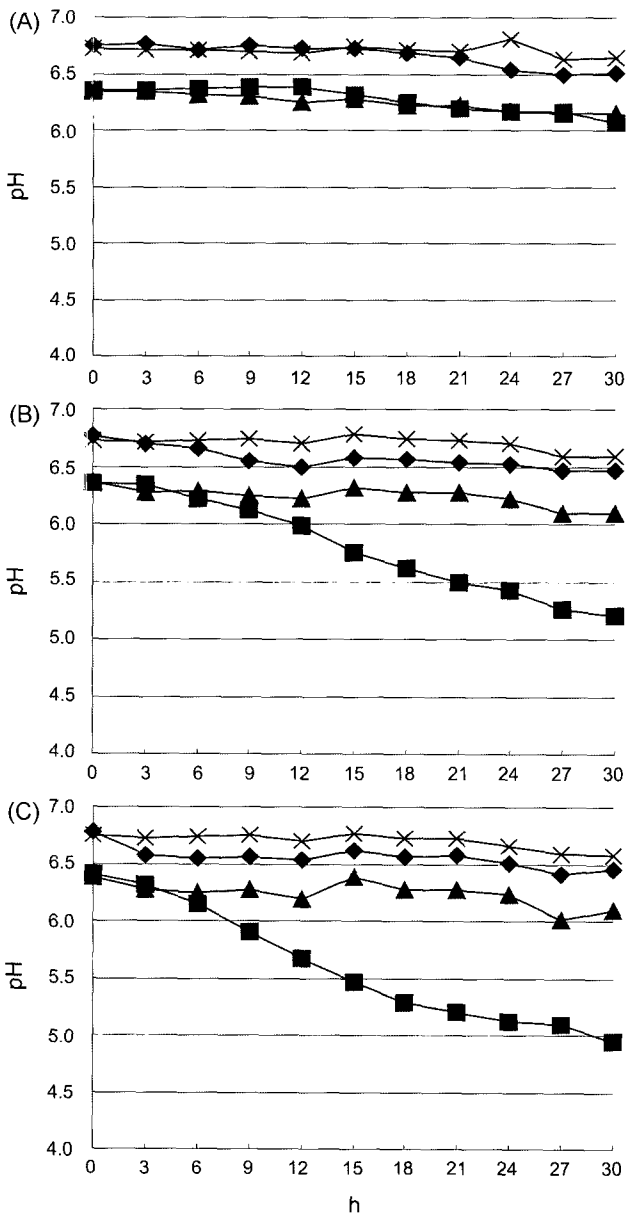

Fig. 7. Change of pH in the culture medium during antibiosis of *Pediococcus* sp. KCA 1303-10 on *Listeria monocytogenes* in soybean soup.

Experiment was performed at 15°C (A), 25°C (B), and 37°C (C). ◆, *L. monocytogenes* without any treatment. ■, *L. monocytogenes* co-cultured with ped+ wild-type strain *Pediococcus* sp. KCA1303-10. ▲, *L. monocytogenes* co-cultured with ped- mutant strain *Pediococcus* sp. KCA1303-10C. ×, *L. monocytogenes* treated with cell-free pediocin K1.

the functions for these genes may help us better interpret our experimental results with the bean soup medium.

The results in the present study for the inhibitory effects of pediocin-producing *Pediococcus* sp. KCA1303-10 against *L. monocytogenes* in the three different media showed that bacteriocin-dependent antibiosis played a more important role at the lower culturing temperature than at the higher temperature, and that the ratio between bacteriocin-dependent and bacteriocin-independent antibiosis was influenced by the medium. In the skim milk system, bacteriocin contributed to the antibiosis against *Listeria* more than non-bacteriocin inhibitory factors such as low pH or organic acids.

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