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Screening of Thermophilic Lactic Acid Bacteria Producing Bacteriocins in the Tropics

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Abstract

Bacteriocin-like activity (BLA) was screened in 50 and 30 strains of thermophilic lactic acid bacteria (LAB) isolated from silages and fermented vegetables, respectively. The BLA of the isolates against some bacterial strains was hardly detected by the paper disk method, while in the isolates of 50 strains (62.5% of all) it was clearly detected by the spot-on-lawn method. Only one strain K-4 isolated from grass silage showed a clear BLA *against Enterococcus faecium* in both methods. The strain belonging to the genus *Enterococcus* produced BLA in the logarithmic growth phase in MRS medium. The BLA was completely inhibited by treatment with trypsin, remained stable under boiling conditions and the molecular weight was about 20,000 based on SDS-PAGE analysis. The BLA produced by this strain was due to bacteriocin and positive against only *Ec. faecium* and *Ec. faecalis*.

Discipline: Food
Additional key words: Enterococcus sp.

Introduction

We have a long history of association with lactic acid bacteria (LAB) and hardly experience any damage due to LAB. Therefore, it is considered that LAB and bacteriocins produced by LAB are generally safe[10]. Actually, a typical bacteriocin "nisin" has already been used in the food industry as a bacteriocidal additive in more than 50 countries of the world. However, the actual use of nisin is limited because of its low solubility in water and lower activity in neutral pH solution. Information on other bacteriocins produced by LAB is limited and bacteriocin except for nisin has not been applied to the food industry.

Therefore, many research groups[2,3,5] have attempted to screen new bacteriocins produced by LAB. In these screenings, LAB were mainly isolated from dairy products in the temperate and frigid zones. However, screening in the tropical zone has not been conducted although some LAB isolated from tropical fermented vegetables and silages may produce new bacteriocins with properties suitable for adaptation to a tropical niche. Therefore, LAB strains that can produce bacteriocin-like activity (BLA) were screened in Thailand, a tropical country.

In this paper, the results of screening conducted in Thailand together with the production of BLA by a selected strain and some of the properties of BLA are described.

Materials and methods

1) Microorganisms

The bacterial strains used in this experiment as

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target strains and their culture conditions are listed in Table 1.

2) Screening

Fifty LAB strains from silages prepared by using plastic bags[14] in Khon Kaen and Bangkok, Thailand, and gathered in Chiang Mai, Thailand, were subjected to the screening. Thirty LAB strains from fermented vegetables bought in the market in Bangkok, Thailand, were also subjected to the screening. These strains were anaerobically isolated by an ordinary plate culture method using an agar plate with Lactobacilli MRS broth (MRS: Difco, USA) containing 1.6% agar and 0.5% $CaCO_3$ at 43°C. For each isolate, the ability to produce BLA was examined by the spot-on-lawn method and the paper disk method.

Spot-on-lawn method[4]: Each isolate was inoculated onto the MRS agar plate to form a colony less than 8 mm in diameter and cultured at 43°C for 24 h, anaerobically. After the culture, the surface of the agar plate (surface with colony formation) was overlaid with a suspension of a target strain at the concentration of ca. 10^6 cfu/mL in soft agar medium and then incubated to grow the target strain for 24 h under favorable conditions as shown in Table 1. Thereafter, the BLA of the isolate was evaluated based on the formation of a clear zone around the colonies. The medium containing 0.7% agar which is suitable for the growth of the target strains (Table 1) was used as a soft agar medium.

Paper disk method[4]: A paper disk (diameter 8 mm, thin type, Toyo Roshi, Japan) with adsorbed 30 μL of cell-free culture filtrate was put on an agar plate containing a target strain at the concentration of ca. 10^6 cfu/mL. Then, the agar plate was incubated under conditions suitable for the growth of the target strain (Table 1). After incubation for 24 h, the BLA of each isolate was evaluated based on the formation of a clear zone around the paper disk. Each isolate was cultured in the MRS broth at 43°C for 20 h and the culture filtrate was adjusted to pH 6.0 with a NaOH solution before adsorption onto the paper disk.

3) Partial purification of the BLA

Partially purified BLA was obtained by ultrafiltration, ammonium sulfate precipitation and ion exchange chromatography. The culture filtrate of strain K-4 using 1 L of MRS broth at 43°C for 12 h under anaerobic conditions was subjected to ultra-filtration by using a Diaflow YM-10 membrane (MW cut off, 10,000) (Amicon Inc., USA), the same operation was repeated after the addition of 200 mL of 20 mM acetate buffer (pH 4.0) and the sample was concentrated to 50 mL after the addition of 20 mM of the same buffer. Thereafter, solid ammonium sulfate was added stepwise for more than 60 min to the concentrate to reach 75% saturation at 4°C and allowed to be stirred for 1 h on a magnetic stir plate. The precipitated suspension was centrifuged for 20 min at 10,000 × g at 4°C and decanted. The pellet was dissolved in 50 mL of the same buffer and dialyzed against distilled water using a

Table 1. Culture conditions and medium for target strains

Strain[a]	Medium[b]	Culture conditions
Enterococcus faecalis IFO 12964	MRS	37°C, 20 h, Anaerobic
Enterococcus faecium IFO 13712	MRS	37°C, 20 h, Anaerobic
Lactobacillus plantarum IFO 14711	MRS	37°C, 20 h, Anaerobic
Lactobacillus plantarum TISTR 541	MRS	37°C, 20 h, Anaerobic
Pediococcus acidilactici TISTR 952	MRS	37°C, 20 h, Anaerobic
Leuconostoc mesenteroides TISTR 473	MRS	37°C, 20 h, Anaerobic
Staphylococcus aureus IFO 15035	NB	37°C, 20 h, Aerobic
Staphylococcus aureus TISTR 029	NB	37°C, 20 h, Aerobic
Salmonella typhimurium TISTR 292	NB	37°C, 20 h, Aerobic
Escherichia coli MAFF 911145	NB	37°C, 20 h, Aerobic
Escherichia coli TISTR 527	NB	37°C, 20 h, Aerobic
Klebsiella pneumoniae NGRI G-1	NB	37°C, 20 h, Aerobic

a): Abbreviations for strain numbers of culture collections are as follows; IFO (Institute for Fermentation, Osaka), TISTR (Thailand Institute of Scientific and Technological Research, Bangkok), MAFF (Ministry of Agriculture, Fisheries and Forestry, Tsukuba), NGRI (National Grassland Research Institute, Nishi-nasuno).

b): Both media were purchased from Difco Laboratories, USA.
MRS; Lactobacilli MRS broth, NB; Nutrient broth.

Spectra/por 1 tube (MW cut off, 6,000–8,000) (Funakoshi Co. Ltd., Tokyo). The dialysate yielded about 2 g of powder by freeze-drying. The powder was dissolved in 10 mL of the same buffer and used for column chromatography (1.6 cm diameter × 40 cm length) with CM Sephadex C-50 (Amersham Pharmacia Biotech Ltd., Sweden) equilibrated with the same buffer. After adsorption of the solution onto the gel, the column was washed with 100 mL of the same buffer and eluted with the same buffer containing 0.8 M sodium chloride. The fraction eluted with the sodium chloride solution was dialyzed against distilled water and yielded 650 mg of powder by freeze-drying. The powder was used for SDS-PAGE analysis and the test of characteristics of BLA.

4) SDS-PAGE

The BLA was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a high cross-linked gel (15%T, 3 × 51 × 58 mm)[8] at 30mA for 2 h. Calibration proteins (Combithek, Boehringer Mannheim Biochemica, Germany) were used as molecular weight markers.

5) Sensitivity to heat, pH and trypsin

The partially purified powder was dissolved in 20 mM acetate buffer (pH 4.0), the pH of the solution was adjusted to 5.0, 6.0 and 7.0 with a NaOH solution and the activity of each pH solution was assayed. The solution prepared in the same manner (pH 6.0) was heated at 100°C for 20 min and the remaining activity was assayed. Furthermore, the same solution was treated with trypsin (Sigma Chemical Co., USA) at a final concentration of 0.5 mg/mL. Samples with or without trypsin were filter-sterilized and incubated at 37°C for 12 h. The remaining activity was assayed. All the BLA was assayed by the paper disk method.

6) Standard line for determining bacteriocin activity

The bacteriocin activity was quantitatively determined by the paper disk method using nisin (1,000 IU/mg, Aplin & Barrett Co., UK) as a standard bacteriocin and *Ec. faecium* IFO 13712 as a target strain. The logarithmic concentration of nisin between 500–10,000 IU/mL gave an essentially straight line against the diameter of the clear zone formed around the paper disk by the target strain. This straight line (referred to as standard line) showed in Fig. 1 was used for determining the bacteriocin activity.

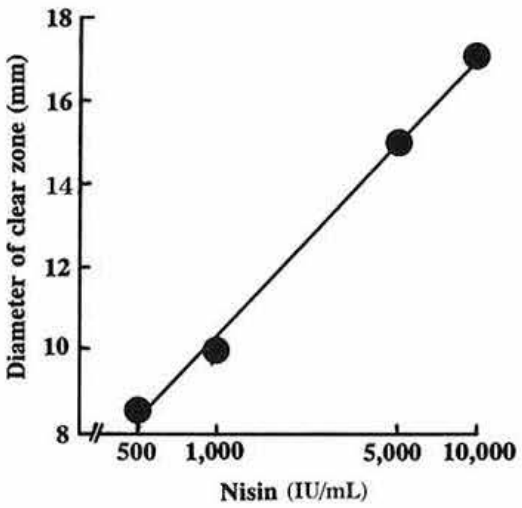

Fig. 1. Standard line for determining bacteriocin activity Nisin and *Enterococcus faecium* IFO 13712 were used as a bacteriocin and a target strain, respectively. The activity was detected by the paper disk method using a certain concentration of nisin.

Results

1) BLA of isolates

Table 2 shows the BLA of typical isolates obtained by the spot-on-lawn method and the paper disk method. Among 50 LAB strains isolated from silages, 20 strains (40% of all) displayed a BLA against some target strains when the spot-on-lawn method was used. All the LAB strains isolated from fermented vegetables displayed a BLA against some target strains when the spot-on-lawn method was used. However, strains found positive by the spot-on-lawn method hardly showed a BLA when the paper disk method was used except for strain K-4. Only the culture filtrate of strain K-4 showed a clear BLA against *Ec. faecium* IFO 13712. Strain K-4 was used for further experiments.

2) Identification of selected strain K-4 at generic level

Bacteriological properties of strain K-4 were examined and the results are summarized in Table 3. The strain was Gram-positive with a typical long chain. The strain grew at 45°C in MRS broth and did not produce gas. The strain also grew in MRS broth with the pH adjusted to 9.6 and NaCl concentration to 6.5%. Based on a manual[6], the strain was tentatively assigned to the genus *Enterococcus*. Therefore, this strain was designated as *Enterococcus* sp. K-4.

3) Spectrum of BLA produced by Enterococcus *sp. K-4*

The spectrum of BLA produced by *Enterococcus* sp. K-4 against 11 bacterial strains is shown in Table 4. The BLA of this strain was positive to only the strains of the genus *Enterococcus* such as *Ec. faecium* IFO 13712 and *Ec. faecalis* IFO 12964 and negative to other strains of the genera *Lactobacillus, Pediococcus, Leuconostoc, Staphylococcus, Salmonella, Escherichia* and *Klebsiella*.

4) Culture conditions of Enterococcus *sp. K-4 for the production of BLA*

Culture temperature: The effect of the culture temperature on the production of bacteriocin in MRS broth was examined as shown in Fig. 2. The maximum bacteriocin acitivity during 20 h of culture was observed at 42–45°C and the activity was 2 times higher than that at 37°C while the maximum lactic acid production was observed at 37–38°C.

Time course of production of BLA: The production of bacteriocin in MRS broth at 43°C was associated with the logarithmic growth of the strain. The highest activity was observed in the maximum growth phase and then the activity gradually decreased as shown in Fig. 3.

Table 2. BLA of typical isolates

Strain No.		Spot-on-lawn method[b]			Paper disk method[b]			Shape[c]
Isolate	TISTR[a]	①	②	③	①	②	③	(Gram stain)
KS-3	1300	++	−	+++	−	−	−	Short rod (+)
4	1301	++	±	+++	−	−	−	Long rod (+)
5	1302	++	±	+++	−	−	−	Long rod (+)
KLD-1	1303	+	−	++	−	−	−	Cocci (+)
5	1304	++	−	+++	−	−	−	Cocci (+)
8	1305	+	−	+++	±	−	−	Cocci (+)
10	1306	+	−	++	−	−	±	Cocci (+)
14	1307	+	−	++	±	−	−	Cocci (+)
K-4	1285	+++	−	++	+	−	−	Cocci (+)
FB 1-7	1308	++	+	++	−	−	−	Long rod (+)
3-5	1309	++	+	++	−	−	−	Cocci (+)
4-5	1310	++	+	++	−	−	−	Short rod (+)
4-6	1311	++	++	+++	−	−	±	Short rod (+)
5-2	1312	++	+	++	−	−	−	Long rod (+)

a): Each strain was deposited in the culture collection of TISTR as described in Table 1.
b): Target strains used were as follows; ① *Ec. faecium* IFO 13712, ② *Ped. acidilactici* TISTR 952, ③ *Leuc. mesenteroides* TISTR 473. Symbols denote the diameter of the clear zone as follows: +++; more than 15 mm, ++; 15–12 mm, +; 12–8 mm, ±; indistinct, −; not detected.
c): (+) denotes Gram-positive reaction.

Table 3. Bacteriological properties of strain K-4

Parameter or test	Reaction or character[a]
Shape	Cocci
Gram stain	+
Gas from glucose	−
Fermentation type	Homo
Growth	
at 45°C	+
at 50°C	−
at 37°C[b]	+
at 37°C[c]	+
at pH 9.6	+
in NaCl 6.5%	+

a): +; Positive, −; Negative.
b): Under anaerobic conditions.
c): Under aerobic conditions.

Table 4. Inhibitory spectrum of BLA produced by *Enterococcus* sp. K-4

Target strain	Inhibition by the culture filtrate[a]
Enterococcus faecalis IFO 12964	+
Enterococcus faecium IFO 13712	+
Lactobacillus plantarum IFO 14711	−
Lactobacillus plantarum TISTR 541	−
Pediococcus acidilactici TISTR 952	−
Leuconostoc mesenteroides TISTR 473	−
Staphylococcus aureus IFO 15035	−
Staphylococcus aureus TISTR 029	−
Salmonella typhimurium TISTR 292	−
Escherichia coli MAFF 911145	−
Escherichia coli TISTR 527	−
Klebsiella pneumoniae NGRI G-1	−

a): +; Positive, −; Negative.

Inoculum size: The effect of the inoculum size at the levels of 10^5, 10^6 and 10^7 cfu/mL was examined for maximum production of bacteriocin in MRS broth at 43°C. As shown in Fig. 4, the culture time for maximum production of the activity decreased with the increase of the inoculum size. The viable cells by inoculation at 10^7 cfu/mL reached maximum counts of 10^9 cfu/mL within 4 h of culture and required only 7 h for maximum bacteriocin activity. Regardless of the inoculum size, the maximum cell counts and bacteriocin activity showed almost the same level (10^9 cfu/mL and 6,200 IU/mL).

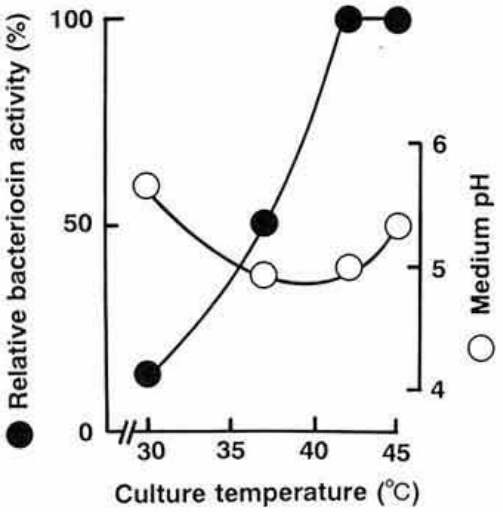

Fig. 2. Effect of culture temperature on the production of bacteriocin by *Enterococcus* sp. K-4
The strain was cultured in MRS broth at various temperatures. The bacteriocin activity of each culture filtrate was detected by the paper disk method, calculated from the standard line and expressed as relative activity.

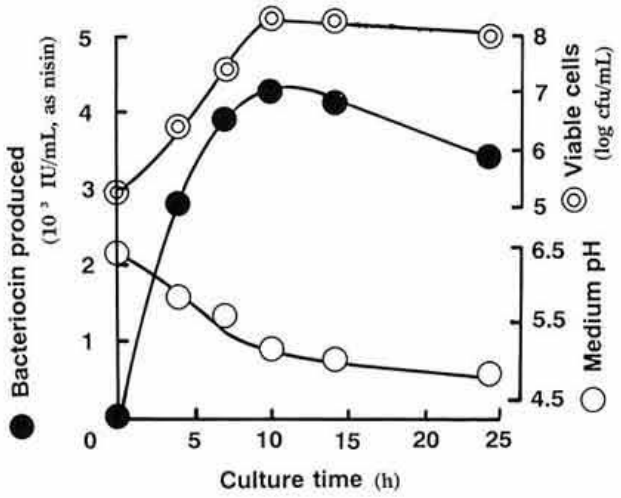

Fig. 3. Typical time course of production of bacteriocin by *Enterococcus* sp. K-4
The strain was cultured in MRS broth at 43°C. The bacteriocin activity was analyzed as indicated in Fig. 2. The viable cells were counted by the agar plate method using MRS broth.

5) Properties of BLA produced by Enterococcus *sp. K-4*

The BLA produced by *Enterococcus* sp. K-4 was stable under boiling conditions (100°C for 20 min) and the level was almost the same at pH 5.0 to 7.0. However, the activity was completely inhibited by treatment with trypsin. The partially purified powder showed a clear BLA zone in the area with an Rf-value of about 0.4–0.5 in SDS-PAGE. The area corresponded to the Rf-value of trypsin inhibitor (MW 20,100) as a standard protein.

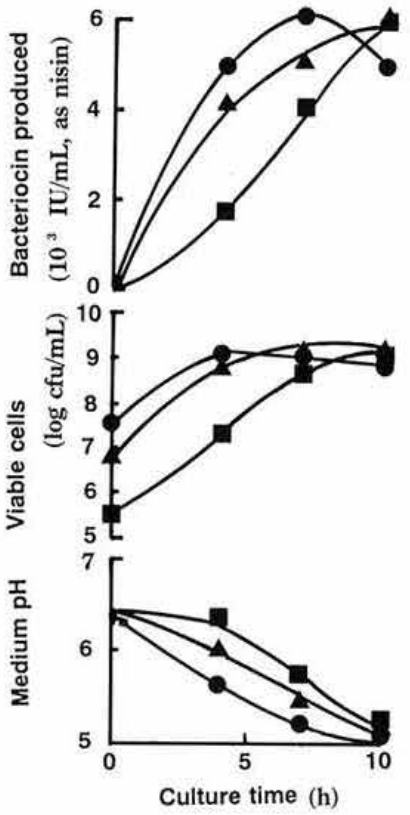

Fig. 4. Effect of inoculum size on the production of bacteriocin by *Enterococcus* sp. K-4
The experimental conditions were the same as those indicated in Fig. 3.
Inoculum size: ■; 5.6×10^5 cfu/mL, ▲; 7.9×10^6 cfu/mL, ●; 5.8×10^7 cfu/mL.

Discussion

Strain K-4, which was isolated from tropical grass silage and belonged to the genus *Enterococcus*, was selected as a bacteriocin-producing strain. When the paper disk method was used, only one strain K-4 showed a clear BLA while 62.5% of the strains among the isolates showed some BLA when the spot-on-lawn method was used in this screening. It is well known that the BLA detected by the spot-on-lawn method can not be reproduced when the paper disk method[10,12] is used. In liquid culture, proteolytic enzymes are released from the producing strain into the culture broth and bacteriocin might be digested by the enzymes. For further detailed studies, the production of BLA in liquid culture is an important and essential factor from the viewpoints of isolation in large quantity and purification of bacteriocin.

The production of bacteriocin by this strain was activated by the temperature during the culture and 42–45°C was the optimum temperature while maximum lactic acid production occurred at 37–38°C as shown in Fig. 2. Generally for the strains of the genus *Enterococcus*, a temperature of 42–45°C during the culture is rather high for bacteriocin production[7,11]. The production of bacteriocin by the current strain at such high temperatures may be due to the origin of the strain which appeared to be a thermophilic strain isolated at 43°C in Thailand. It is possible that the bacteriocin produced by this strain showed properties suitable for adaptation to a tropical niche. The production of bacteriocin by this strain occurred during the logarithmic growth phase and the inoculum size did not increase the amount of bacteriocin production, while the culture time was shortened for maximum production. When the inoculum size increased, maximum bacteriocin production and viable cell counts were 6,200 IU/mL and 10^9 cfu/mL, respectively. To increase the bacteriocin production, it is necessary to increase the cell density in the medium together with the modification of the medium composition.

The BLA produced by strain K-4 was positive against only the strains of the genus *Enterococcus*. Usually, bacteriocins show a narrow inhibitory spectrum and give a positive reaction to the same and/or related genus species[9]. This tendency was also observed in the current study since strain K-4 which belonged to the genus *Enterococcus* was active only against *Ec. faecium* and *Ec. faecalis*. The BLA of this strain was thermo-tolerant and was detected in solutions with a neutral and acidic pH. The apparent molecular weight of main bacteriocin was about 20,000 in SDS-PAGE (data not shown). Some bacteriocins produced by the strains of the genus *Enterococcus* have been reported by several research groups[1,7,11,13,15]. These bacteriocins had a molecular weight of less than 10,000 and were active against *Listeria monocytogenes*. Further studies should be carried out for the purification and analysis of the chemical properties of the new bacteriocin produced by this strain.

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