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Production of Bacteriocins by Several Lactic Acid Bacteria and Their Application to Growth Inhibition of Spoilage Bacteria Related to Hiochi

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We investigated the antimicrobial activity of several bacteriocins, which were produced by newly isolated lactic acid bacteria in koji extract medium supplemented with rice protein hydrolyzate (RPH), against strains of NBRC type culture and spoilage bacteria isolated from deteriorated sake. The partial sequences of the 16S rRNA gene of the spoilage bacteria isolated from sake showed a high similarity to those of *Lactobacillus fructivorans, Lactobacillus hilgardii*, and *Lactobacillus paracasei*, with 99.6-100% identity. *Enterococcus durans* C102901 (C102901), *Lactococcus lactis* subsp. *lactis* C101910 (C101910), and *Lactococcus lactis* subsp. *lactis* NBRC 12007 (NBRC 12007) grew well and produced bacteriocins with high activity in koji extract medium supplemented with RPH. When culture supernatants containing bacteriocins from C101910 and NBRC 12007 were added to the medium at a volume ratio of 10% (v/v), the growth of *L. fructivorans* NBRC 13954^T was significantly inhibited and the viable cell concentration decreased below the detection limit (1.0×10^2 cells/ml) at 4 h. Further, by the addition of bacteriocin solutions from C102901, C101910, and NBRC 12007 to the medium at a volume ratio of 1% (v/v), the growth of *L. hilgardii* NBRC 15886^T and H130 (closely related to *L. hilgardii*) isolated from putrid sake was bactericidally inhibited and the colony-forming units fell by more than three orders of magnitude within 4-12 h as compared with the initial cell concentration.
Key words: bacteriocin, hiochi bacteria, *Lactobacillus*, koji extract medium, rice protein

1. Introduction

In the food industry, contamination by spoilage microorganisms results in severe safety and economic problems. In the sake-brewing process, spoilaging phenomena caused by several *Lactobacillus* species, so-called hiochi bacteria, seriously deteriorate the quality of sake, such as lowering the pH, increasing turbidity, and producing off-flavors [1,2]. Sake with an ethanol concentration greater than 15% tends to be more resistant to spoilage by microorganisms during storage and aging, but alcoholophilic and alcohol-tolerant hiochi bacteria can grow in such an alcoholic environment and consequently spoil sake. To prevent spoilage by an outbreak of hiochi bacteria in the sake-making process, pasteurization has been carried out at a low temperature (about 65℃) for a short time (2-3 min) or a microfiltration device has been used to remove hiochi bacteria; however, due to incomplete inactivation and insufficient removal of hiochi bacteria, they frequently deteriorate the quality of sake, especially non-pasteurized (fresh) sake, during storage and distribution.

Bacteriocins produced by lactic acid bacteria (LAB), which are generally recognized as safe, are of increasing interest because of their antimicrobial spectra with feasible application to foods such as meat and dairy products, fruits, vegetables, cereals, and beverages [3-5]. Since bacteriocins are generally heat-stable, the combination use of bacteriocins with heat treatment is expected to facilitate the complete inactivation of hiochi bacteria and reduce the cost of the heating operation [6,7]; however, since the use of bacteriocins as food additives demands an exhaustive evaluation of their toxicological effects before legal acceptance, bacteriocins are not extensively used in the food industry. Nisin A produced by *Lactococcus lactis* subsp. *lactis*, is the only bacteriocin commercially exploited to date, because its use as a biopreservative has been widely investigated in a large variety of fresh and processed foods

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[8, 9]. To be approved for use as a food preservative, many isolated bacteriocins (pediocin, lacticin, enterocin, *etc.*) other than nisin A should be fully characterized.

The inhibitory effect of commercially pure nisin A on the growth of hiochi bacteria was confirmed by Kanatani *et al.* [1]. They showed the sensitivity of hiochi bacteria to nisin A using the agar diffusion method. We previously isolated a novel bacteriocin-producing bacterium, *Staphylococcus* sp. NPSI 38 (NPSI 38: referred as *Pediococcus* sp. NPIB-38 in the ref.), from rice koji [10]. We found that the bacteriocin showed antimicrobial activity against selected strains of hiochi bacteria (*Lactobacillus fructivorans, Lactobacillus hilgardii, etc.*). We also reported that NPSI 38 produced bacteriocin in koji extract medium supplemented with rice protein hydrolyzate (RPH) and that the culture supernatant containing the bacteriocin was found to be effective for inhibiting the growth of *L. hilgardii*, a representative hiochi bacterium [11]. To exploit novel bacteriocins with inhibitory activity against hiochi bacteria, further investigations are necessary for not only the production of bacteriocin by newly isolated LAB but also the inhibitory effects of bacteriocins formed on the growth of hiochi bacteria.

In this study, we isolated spoilage bacteria from sake breweries in Niigata Prefecture and compared the similarity of partial sequences of the 16S rRNA gene (16S rDNA) with related strains using a database. We also investigated bacteriocin production by several newly isolated LAB using koji extract medium supplemented with RPH. Further, to evaluate the antimicrobial activity of bacteriocins against spoilage bacteria, we examined the inhibitory effects of bacteriocin solutions produced on the growth of spoilage bacteria containing strains isolated from putrid sake.

2. Materials and Methods

2.1 Media and microorganisms

SI medium (commercial medium for detecting hiochi bacteria [12], Brewing Society of Japan, Tokyo) with 10% ethanol (pH 5.0) was used to isolate putrid bacteria from sake spoiled in Niigata Prefecture. MRS medium [13] containing 10 g/l glucose (pH 6.8) was used to cultivate all bacteria, except for *L. hilgardii* NBRC 15886^T, and two strains of H82 and H119 isolated from spoiled sake, which could not grow in MRS medium but in SI medium. *L. hilgardii* NBRC 15886^T was also cultivated in modified MRS medium with 10 g/l glucose (pH 6.8) from which Tween 80 and diammonium hydrogen citrate were omitted as described previously [11]. Bacterial strains were maintained at −80℃ in 25% glycerol.

Lactobacillus gasseri NPSI 240 (NPSI 240), *Enterococcus durans* L28-1 (L28-1), *Enterococcus durans* C102901 (C102901), *Lactococcus lactis* subsp. *lactis* C101910 (C101910), and *Lactococcus lactis* subsp. *lactis* NBRC 12007 (NBRC 12007), a nisin A producer, were used as bacteriocin-producing bacteria. NPSI 240 was isolated from rice koji by Kaneoke *et al.* [10], and L28-1, C102901, and C101910 from soil and lake water by Yanagida *et al.* [14-16]. *L. fructivorans* NBRC 13954^T, *L. hilgardii* NBRC 15886^T, and *Lactobacillus paracasei* subsp. *paracasei* NBRC 15889^T were used as indicator microorganisms. Eighty-eight strains of bacteria were isolated from turbid sake spoiled during storage. Of the isolated bacteria, four strains (H28, H82, H119 and H130) listed in Table 3 were also used as indicator microorganisms.

2.2 16S rRNA gene (16S rDNA) sequencing of isolated bacteria and homology analysis

Eighty-eight strains of bacteria isolated from spoiled sake were cultivated in SI medium with 10% ethanol. The total genomic DNAs of the bacteria were extracted from cells by InstaGene Matrix (Biorad, CA, USA) and used as polymerase chain reaction (PCR) templates. Partial 16S rDNAs of the bacteria were amplified by PCR using four sets of primers listed in Table 1 [17], which correspond to partial positions of *Escherichia coli* 16S rDNA. PCR was performed in reaction mixture consisting of 10 μl of template DNA, pure Taq ready-to-go PCR beads (Amersham Biosciences, NJ, USA), and 0.25 μl (5 pmol) of each primer using a thermal cycler (MP; Takara, Otsu). PCR conditions were as follows: hot start at 95℃ for 5 min, followed by 30 cycles of 95℃ for 15 s, 60℃ for 30 s, and 68℃ for 1 min. DNA products were purified using a PCR Clean-up Kit (Mo Bio Laboratories, CA, USA) and then amplified using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, CA, USA) and a sequence primer, -21M13 (5'-TGTAAAACGACGGCCAGT-3'). The amplified products were sequenced with an ABI PRISM 377 DNA sequencer (Applied Biosystem). A homology search of the partial sequences of 16S rDNA obtained was performed using a BLAST search of the DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/).

2.3 Determination of antimicrobial activity

Antimicrobial activity was determined by the agar diffusion method using a stainless cup according to the procedure reported previously [11]. The agar concentration of each medium was adjusted to 1%. The supernatant pre-

Table 1 Oligonucleotide primers used in this study[a].

Primer	Position[b]	Sequence (5'-3')[c]
M27F	11-27	TGTAAAACGACGGCCAGTAGAGTTTGATCCTGGCTCAG
520R	531-517	ACCGCGGCKGCTGGC
27F	11-27	AGAGTTTGATCCTGGCTCAG
M350R	361-342	TGTAAAACGACGGCCAGTCTACTGCTGCCTCCCGTAG
1080F	1074-1094	GTYGTGARATGTTGGGTTAAG
M1392R	1410-1392	TGTAAAACGACGGCCAGTTITGACGGGCGGTGTGTAC
M1094F	1077-1094	TGTAAAACGACGGCCAGTGTGARATGTTGGGTTAAG
1522R	1541-1522	AAGGAGGTGATCCARCCGCA

[a]Oligonucleotide primers reported by Mori *et al.* [17].
[b]16S rRNA gene position of *E. coli*.
[c]K=G or T; R=A or G; Y=C or T; I=Inosine.

pared by centrifugation (at 15,500 rpm for 10 min) of culture broth obtained after cultivation of bacteriocin-producing LAB was analyzed for antimicrobial activity. In the cultivation to prepare the supernatant, MRS medium was used for NPSI 240, L28-1, and NBRC 12007, and a corresponding suitable medium described previously [15, 16] was used for C102901 and C101910. Antimicrobial activity was determined by measuring the diameter of the growth-inhibitory zone around the cup after incubation. One unit of antimicrobial activity was defined as the amount of bacteriocin which showed a growth-inhibitory zone diameter equal to that obtained by 1 ng of pure commercial nisin A (Sigma Chemical Co., St. Louis, USA) standard solution as reported previously [11].

2.4 Bacteriocin production

Bacteriocin production was carried out in a medium bottle using MRS medium, 10% (v/v) koji extract medium, and 10% (v/v) koji extract medium supplemented with RPH. The koji extract solution and RPH (Shimada Kagaku, Nagaoka) were prepared as described previously [11]. Bacteriocin-producing LAB were stationarily cultivated at 30℃ for 48 h at an initial turbidity of 0.1-0.2 at 660 nm (T_{660}=0.1-0.2).

2.5 Growth-inhibitory activity of bacteriocin solution

Three NBRC-type strains and the isolated spoilage bacteria were cultivated in MRS, modified MRS or SI medium suitable for each indicator microorganism. The bacteriocin solution was prepared by cultivating bacteriocin-producing LAB in koji extract medium supplemented with RPH at a protein concentration of 40 g/l. The culture supernatant was obtained by centrifugation (15,500 rpm, 10 min) of the culture broth. The resultant supernatant was sterilized by filtration using a membrane of 0.20 μm pore size (Dismic 25CS020AS, Advantec Toyo Co., Tokyo) and used as a bacteriocin solution. Each indicator microorganism was inoculated in a medium containing 1 or 10% (v/v) of bacteriocin solution and incubated at 30℃ for 24 h. Antimicrobial activity was 1.8-3.5×10 and 1.8-3.5×10^2 U/ml for media containing 1 and 10% (v/v) of bacteriocin solution, respectively. The inhibitory effect of each bacteriocin solution on the cell growth of indicator microorganisms was evaluated by measuring the concentration of viable cells as described below.

2.6 Other analytical methods

Cell concentration was determined by measuring T_{660}. The number of viable cells was counted by the plate culture method using SI, MRS, or modified MRS agar medium containing 10 g/l glucose. Viable cell concentration was expressed as colony-forming units per milliliter (CFU/ml). Protein concentration was determined by the method of Lowry *et al.* [18] using bovine serum albumin as a standard. The supernatant obtained by centrifugation (15,500 rpm, 10 min) of culture broth was analyzed for total soluble sugar, glucose, and lactic acid concentrations. The concentrations of total soluble sugar, glucose, and lactic acid were measured as described previously [19, 20].

3. Results

3.1 Isolation and 16S rDNA sequencing of spoilage bacteria

Eighty-eight spoilage bacteria were isolated from a large number of deteriorated sake using SI medium with 10% ethanol. The partial 16S rDNAs of spoilage bacteria were sequenced and analyzed. Table 2 shows the similarity of partial 16S rDNA sequences between 88 strains of spoilage

bacteria and related bacteria. Of the isolated bacteria, the sequences of 71 strains showed a high similarity to those of *L. fructivorans* with more than 99.6% identity. The sequences of 14 strains and three strains were identical to those of *L. paracasei* and *L. hilgardii*, respectively. Apart from the detection frequency from spoiled sake, the species of genus *Lactobacillus* isolated in this study were in fair agreement with those reported in previous papers [2, 21-24].

3.2 Antibacterial spectrum

Preliminary experiments showed that five strains of LAB were potential bacteriocin producers (data not shown). Each culture supernatant of the five strains was prepared and used to measure antimicrobial activity against indicator microorganisms (three NBRC type strains and four typical strains isolated from spoiled sake: H28, H82, H119, and H130). On the basis of data on the partial sequence of 16S rDNA described above, H82 and H119 belonged to Group 1a and 1b, respectively and H28 and H130 belonged to Group 2 and Group 3, respectively, as shown in Table 2. Table 3 shows the antimicrobial activities of bacteriocins produced by LAB. The bacteriocin from *L. gasseri* NPSI 240 slightly inhibited the growth of *L. hilgardii* NBRC 15886^T and H130. L28-1 and C102901 produced bacteriocins with high antimicrobial activity against *L. hilgardii* NBRC 15886^T and H130. Bacteriocins produced by C101910 and NBRC 12007 showed a relatively broad inhibitory spectrum against indicator bacteria. In particular, high antimicrobial activities against *L. hilgardii* NBRC 15886^T, *L. fructivorans* NBRC 13954^T, and H130 were observed for bacteriocins produced by C101910 and NBRC 12007, although the bacteriocins did not show any antimicrobial activity against *L. paracasei* subsp. *paracasei* NBRC 15889^T.

Table 2 Sequence similarities of 16S rRNA gene between bacteria isolated from spoiled sake and related standard bacteria.

Group	Number of strains	Sequence length compared (bp)	Closest relatives	Accession No. of relative	Homology (%)
1a	61	808	*Lactobacillus fructivorans*	X76330	99.9[a]
1b	10	808	*Lactobacillus fructivorans*	X76330	99.6[b]
2	14	851	*Lactobacillus paracasei*	DQ486146	100
3	3	315[c]	*Lactobacillus hilgardii*	AB368911	100

[a]Base of position 1152 corresponding to that of *E. coli* was unknown, different from A for the closest bacterium.
[b]Bases of positions 1152, 1171 and 1445 corresponding to those of *E. coli* were G, G, and C, different from A, A, and T for the closest bacterium, respectively.
[c]Partial 16S rDNAs of the bacteria belonging to Group 3 were amplified by the PCR using only two sets (1080F and M1392R; M1094F and 1522R) of primers.

Table 3 Antimicrobial spectrum of bacteriocin produced by LAB against bacteria related to hiochi.

Indicator strains	Medium	Antimicrobial activity[a]				
		NPSI 240	L28-1	C102901	C101910	NBRC 12007
L. fructivorans NBRC 13954^T	MRS	-	-	-	+++	+++
H82[b]	SI	-	-	-	+	++
H119[b]	SI	-	-	-	++	++
L. paracasei subsp. *paracasei* NBRC 15889^T	MRS	-	-	+	-	-
H28[c]	MRS	-	-	-	+	+
L. hilgardii NBRC 15886^T	mMRS[e]	+	++	+++	+++	+++
H130[d]	MRS	+	++	+++	+++	+++

[a]Diameter of growth-inhibitory zone ; +++: > 25 mm; ++: 15~25 mm; +: < 15 mm; -: no inhibition
[b]H82 and H119 belong to Group 1a and 1b in Table 2, respectively.
[c]H28 belongs to Group 2 in Table 2.
[d]H130 belongs to Group 3 in Table 2.
[e]Modified MRS medium.

3.3 Bacteriocin production by selected LAB using koji extract medium

We previously exploited the procedure for bacteriocin production using koji extract medium supplemented with RPH as an alternative to natural ingredients (polypeptone, meat extract, and yeast extract) in MRS medium, which can not be lawfully used in the sake-producing process. We attempted bacteriocin production using five selected strains with koji extract medium supplemented with RPH. Figures 1 and 2 show the results of bacteriocin production

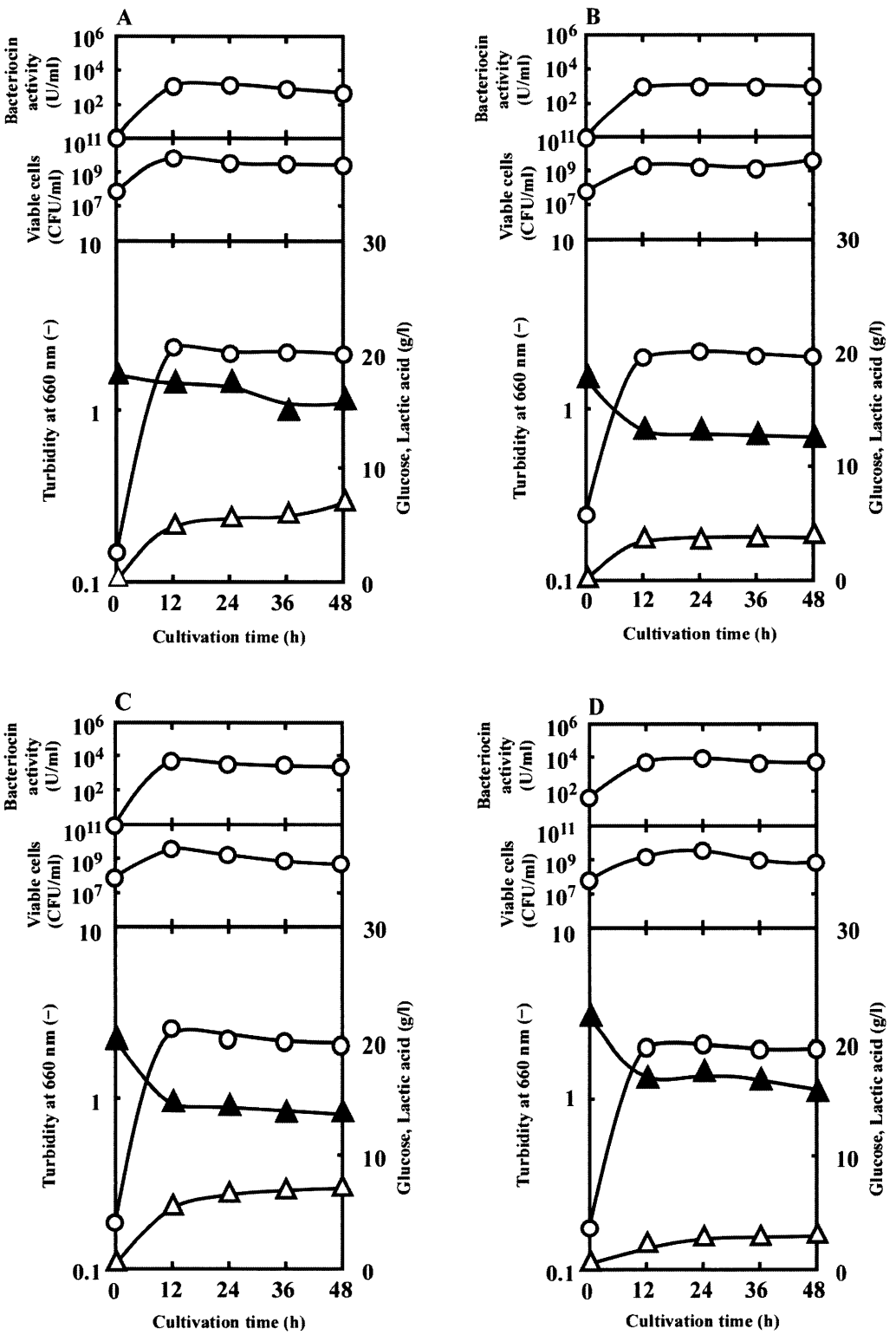

Fig. 1 Bacteriocin production using MRS medium by *E. durans* L28-1 (A), *E. durans* C102901 (B), *L. lactis* subsp. *lactis* C101910 (C), and *L. lactis* subsp. *lactis* NBRC 12007 (D). Symbols: open circles, bacteriocin activity, viable cells, and turbidity; closed triangles, glucose; open triangles, lactic acid.

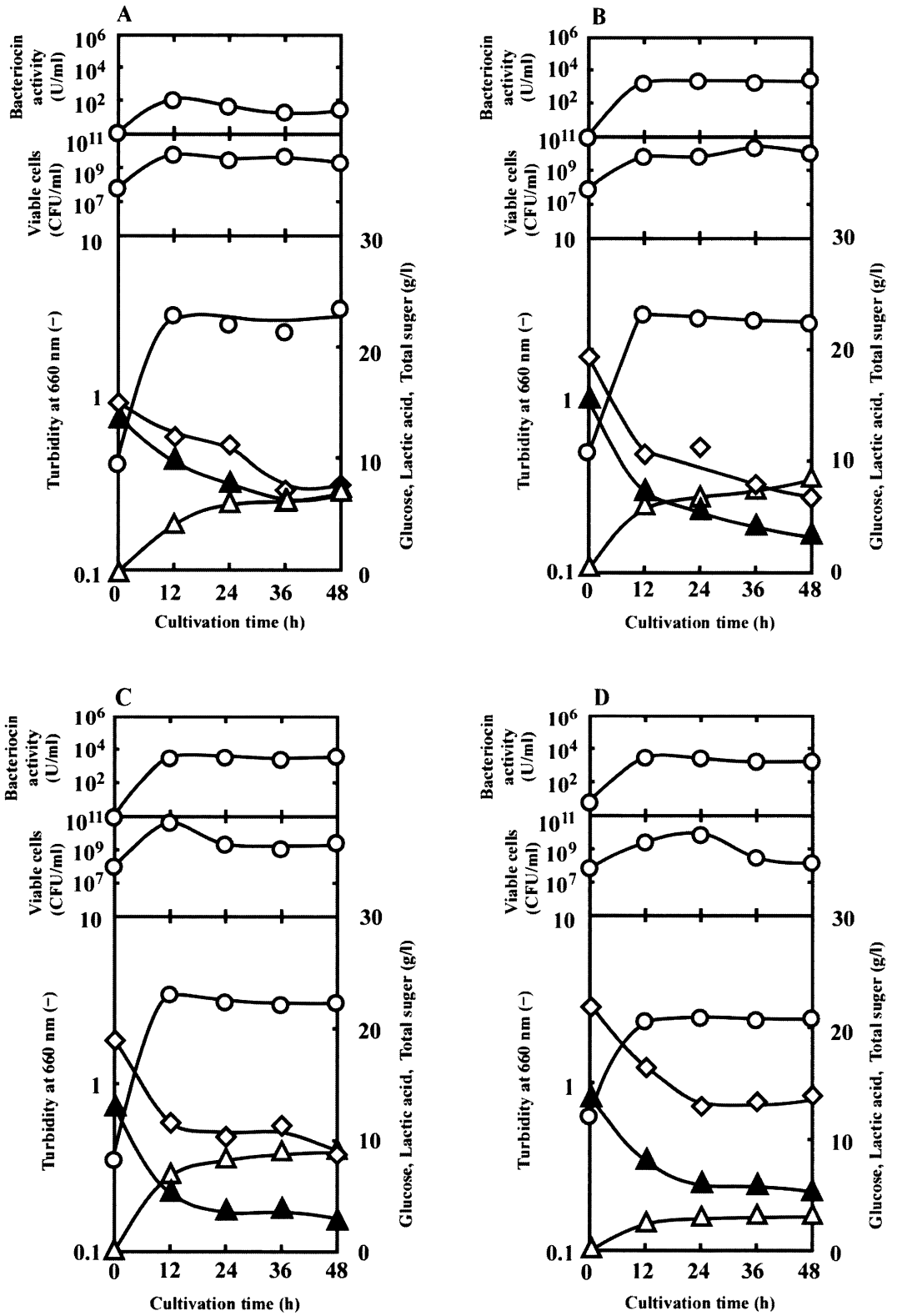

Fig. 2 Bacteriocin production using koji extract medium supplemented with rice protein hydrolyzate by *E. durans* L28-1 (A), *E. durans* C102901 (B), *L. lactis* subsp. *lactis* C101910 (C), and *L. lactis* subsp. *lactis* NBRC 12007 (D). Symbols: open circles, bacteriocin activity, viable cells, and turbidity; closed triangles, glucose; open triangles, lactic acid; open diamond, total sugar.

by LAB using MRS medium with 20 g/l glucose and the koji extract medium supplemented with RPH at a protein concentration of 40 g/l, respectively. When MRS medium was used, NPSI 240 slightly produced bacteriocin (8.2 U/ml) (data not shown). As shown in Figure 1, the results of turbidities and viable cell concentrations indicated that the cells of L28-1, C102901, C101910, and NBRC 12007 grew vigorously in the initial stage, followed by a stationary phase. The decreased growth rate seemed not to be caused by the depletion of nutrients in the media, but by the lowering of pH due to the accumulation of lactic acid, similarly to other organic acid-producing bacteria described previously [19, 20, 25]. In MRS medium, the bacteriocin was produced by L28-1, C102901, C101910, and NBRC 12007 in a concentration range of 1.1×10^3 to 1.1×10^4 U/ml, depending upon the strain used. When koji extract medium was used alone, little or no growth of all LAB was observed. C101910 only slightly produced bacteriocin (3.7×10 U/ml) and the other four LAB produced negligible concentrations of bacteriocins (Table 4). In the koji extract medium supplemented with RPH, NPSI 240 produced little or no bacteriocin (data not shown) and the concentrations of bacteriocin produced by L28-1 were 1.3×10^2 and 5.3×10 U/ml at a cultivation time of 12 and 24 h, respectively. The low antimicrobial activity at 24 h is probably due to partial hydrolysis of the bacteriocin produced by proteases secreted in the medium. The activities of bacteriocin produced by L28-1 are much lower than those (about 1.5×10^3 U/ml) using MRS medium, in spite of the good growth, as shown in Fig. 2A; however, the cells of C102901, C101910, and NBRC 12007 grew almost identically to those using MRS medium and consequently they produced bacteriocin at a high concentration.

Table 4 shows a comparison of bacteriocin production by LAB using different media. The concentration of bacteriocin was significantly dependent upon the kind of medium tested and the strain of LAB used as a bacteriocin producer. When koji extract medium supplemented with RPH

Table 4 Bacteriocin production by LAB using koji extract medium with rice protein hydrolyzate.

Medium	Bacteriocin concentration (U/ml)[a]				
	NPSI 240	L28-1	C102901	C101910	NBRC 12007
MRS	8.2	1.5×10^3	1.1×10^3	3.6×10^3	1.1×10^4
Koji extract	ND[a]	ND	ND	3.7×10	ND
Koji extract + Rice protein	ND	5.3×10	1.8×10^3	3.5×10^3	2.8×10^3

[a]Bacteriocin activity was determined for culture supernatant obtained at a cultivation time of 24 h.
[b]ND: Not detected.

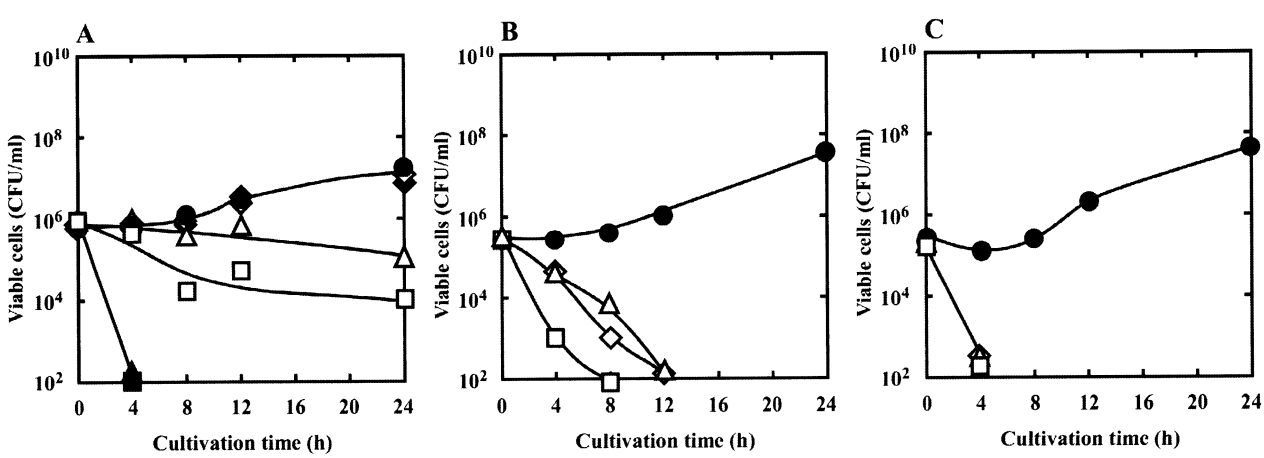

Fig. 3 Inhibitory effect of bacteriocins from LAB on the cell growth of *L. fructivorans* NBRC 13954^T (A) in MRS medium, *L. hilgardii* NBRC 15886^T (B) in modified MRS medium, and H130 (C) in MRS medium. Bacteriocin solution was added to the media at a volume ratio at 1% (v/v) or 10% (v/v). Viable cell concentrations of bacteria in culture broth with bacteriocin from *E. durans* C102901 (1.8×10 U/ml=1% (v/v), open diamond; 1.8×10^2 U/ml=10% (v/v), closed diamond), bacteriocin from *L. lactis* subsp. *lactis* C101910 (3.5×10 U/ml=1% (v/v), open triangles; 3.5×10^2 U/ml=10% (v/v), closed triangles), or bacteriocin from *L. lactis* subsp. *lactis* NBRC 12007 (2.8×10 U/ml=1% (v/v), open squares; 2.8×10^2 U/ml=10% (v/v), closed squares) are shown. Closed circles show the results for viable cell concentrations of bacteria in control culture broth without bacteriocin.

was used, NPSI 240 and L28-1 produced little or no bacteriocin but C102901, C101910, and NBRC 12007 produced bacteriocin at a concentration higher than 1.8×10^3 U/ml. The values for C102901 and C101910 were almost as high as the concentrations in the cultivation using MRS medium, although the concentration of bacteriocin by NBRC 12007 decreased to about one-fourth of that using MRS medium. In our previous study, we reported that the concentration of bacteriocin by NPSI 38 in koji extract medium supplemented with RPH was 6.8×10 U/ml. Using newly isolated LAB (C102901 and C101910) as bacteriocin producers, bacteriocins showing antimicrobial activity more than 25-fold that produced by NPSI 38 could be obtained.

3.4 Inhibitory effect of bacteriocins on growth of hiochi bacteria

The growth-inhibitory effect was examined using a bacteriocin solution prepared by cultivating bacteriocin-producing LAB (C102901, C101910, and NBRC 12007) in koji extract medium with RPH. Figure 3 shows antimicrobial activity against spoilage bacteria (*L. fructivorans* NBRC 13954^T, *L. hilgardii* NBRC 15886^T, and H130). In bacteriocin-free cultivation, all indicators grew gradually and cell concentrations reached 10^7 to 10^8 CFU/ml. When bacteriocin solution was added to medium at a volume ratio of 1% (v/v), the growth of *L. fructivorans* NBRC 13954^T was significantly inhibited and the viable cell concentration at 24 h decreased by a factor of about 10 for bacteriocin from C101910 and about 100 for bacteriocin from NBRC 12007 as compared with the initial value. In contrast, bacteriocin from C102901 showed no inhibitory effect on the growth of *L. fructivorans* NBRC 13954^T during cultivation. When the volume ratio of the bacteriocin solution to the medium was 10% (v/v), the number of viable cells of *L. fructivorans* NBRC 13954^T fell by more than four orders of magnitude within 4 h for bacteriocins from C101910 and NBRC 12007 as compared with the initial concentration; however, bacteriocin from C102901 at a higher concentration exhibited no growth-inhibitory activity against *L. fructivorans* NBRC 13954^T during cultivation. On the other hand, in media containing 1% (v/v) of bacteriocin solutions, the number of viable cells of *L. hilgardii* NBRC 15886^T fell by more than three orders of magnitude within 8 h for bacteriocin from NBRC 12007, and 12 h for bacteriocins from C102901 and C101910, respectively, as compared with the initial concentration. Furthermore, H130 was the most sensitive to the bacteriocins and the number of viable cells decreased by more than three orders of magnitude within 4 h for every bacteriocin; however, the bacteriocin solutions tested showed only a slight growth-inhibitory effect on H82 and H119, and negligible growth-inhibitory effect on *L. paracasei* subsp. *paracasei* NBRC 15889^T and H28 listed in Table 3 (data not shown), respectively, even when bacteriocin solutions were added to medium at a volume ratio of 10% (v/v).

4. Discussion

The partial 16S rDNA sequences of the most dominant spoilage bacteria isolated from deteriorated sake using SI medium with 10% ethanol showed the highest similarity to *L. fructivorans* with more than 99.6% identity. The other spoilage bacteria possessed identical partial 16S rDNA sequences to those of *L. paracasei* and *L. hilgardii* in the database, as shown in Table 2. These results were similar to previous works in which hiochi bacteria were identified as several *Lactobacillus* species (*L. fructivorans*, *L. paracasei* subsp. *paracasei*, *L. hilgardii*, and *Lactobacillus rhamnosus*) by molecular biological and biochemical methods [21-23]. Recently, Wada and Mizoguchi pointed out that only *L. fructivorans* has been isolated from spoiled sake [2]; however, to our knowledge, few statistical studies on the emerging frequency of species of hiochi bacteria in spoiled sake have been reported [22]. In this study, we could estimate that the major species of *Lactobacillus* causing hiochi in Niigata Prefecture were *L. fructivorans*, *L. paracasei*, and *L. hilgardii*.

To prevent hiochi from occurring in the sake-brewing industry, novel bacteriocins with antimicrobial activity against the strains of *Lactobacillus* described above have to be exploited. Therefore, we focused our attention on the application of bacteriocins produced by newly isolated LAB to growth inhibition against bacteria related to hiochi. Thus, we found that the bacteriocin (durancin L28-1A) produced by L28-1 is 3.4 kDa in molecular size and is a new natural enterocin variant [14]. We also reported that the bacteriocin from C102901 was found to be different from durancin L28-1 in terms of molecular size and N-terminal amino acid and that C102901 can produce the bacteriocin in a poor nutrient medium [15]. Further, we found that C101910 can produce nisin Z in a simple medium at a low temperature, although nisin Z itself is a well-known bacteriocin [16]. Recently, considering the application of bacteriocin to the sake-brewing process, we reported bacteriocin production by newly isolated NPSI 38 using koji extract medium supplemented with RPH [11]. Bacteriocin production by newly isolated LAB using koji

extract medium supplemented with RPH is an important problem. As shown in Figs. 1 and 2, the concentration of bacteriocin produced by C102901 and C101910 in koji extract medium supplemented with RPH was almost as high as the concentration in MRS medium. However, when the former medium was used, L28-1 produced a slight amount (5.3×10 U/ml) of bacteriocin and the concentration of bacteriocin produced by NBRC 12007 was high, but approximately 25% that in MRS medium. The results are consistent with the results in our previous reports [15, 16], showing that C102901 and C101910 were isolated from the rhizosphere of trees and lake water, respectively, and could grow in simple medium with poor nutritional constituents, as described above. Consequently, we successfully produced three kinds of bacteriocin from C102901, C101910, and NBRC 12007 using medium which can be legally used in the sake-brewing process.

In experiments on the evaluation of growth-inhibitory activity, except for the addition of bacteriocin solution from C102901 to *L. fructivorans* NBRC 13954^T, the addition of bacteriocin solutions resulted in a decreased number of viable cells, as shown in Fig. 3, suggesting bactericidal activity against *L. fructivorans* NBRC 13954^T, *L. hilgardii* NBRC 15886^T, and H130. Although high antimicrobial activity of bacteriocins from C101910 and NBRC 12007 against *L. fructivorans* NBRC 13954^T was observed in the agar diffusion assay (Table 3), those bacteriocins showed lower growth-inhibitory activity against *L. fructivorans* NBRC 13954^T in MRS medium as compared with against *L. hilgardii* NBRC 15886^T and H130 (Fig. 3). The discrepant results seem to be caused by the difference in antimicrobial action between solid medium with agar and liquid medium. The influence of growth environment, such as the form and constituents of medium, pH, and temperature, on the action mode of bacteriocins is very interesting but still unclear [9, 26]. We previously reported that the bacteriocin produced by NPSI 38 showed bacteriostatic activity against *L. hilgardii* NBRC 15886^T [11]. In this study, we could obtain bacteriocins with a mode of action different from the bacteriocin from NPSI 38. We expect a synergistically inhibitory effect of bacteriocins with different modes of action on the growth of bacteria related to hiochi. The investigation of synergistic antimicrobial activity is considered to be an interesting subject of our future research. In addition, in this study, the bacteriocins tested exhibited no inhibitory effect on the growth of other spoilage bacteria (H82 and H119 closely related to *L. fructivorans*) listed in Table 3 (data not shown); that is, the antimicrobial action of the some bacteriocins was confirmed to be not species-specific but strain-specific, as reported previously [10, 27, 28]; therefore, from the viewpoint of practical utilization, it is necessary to clarify the minimum bacteriocin concentration required to exhibit complete growth inhibition in liquid medium against each spoilage bacterium. We are now focusing on investigating the inhibitory effect of the initial concentration of bacteriocin on the growth of putrid bacteria listed in Table 3 and the application of bacteriocins from C102901, C101910, and NBRC 12007 in the seed mash-making stage (Moto) in the sake-brewing process.

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◇◇◇ 和文要約 ◇◇◇

乳酸菌によるバクテリオシンの生産とそれらの火落ち関連腐敗細菌の増殖抑制への応用

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我々は米タンパク質加水分解物（RPH）を添加した麹汁培地を用いて，新奇に単離した乳酸菌によるバクテリオシン生産について検討した．また，生産されたバクテリオシンの清酒の腐敗に関連する細菌（火落菌）に対する抗菌活性についても検討した．清酒より単離した火落菌の16S rRNA遺伝子の部分塩基配列は，*Lactobacillus fructivorans, Lactobacillus hilgardii* および *Lactobacillus paracasei* と高い相同性を示した．バクテリオシン生産菌 *Enterococcus durans* C102901（C102901），*Lactococcus lactis* subsp. *lactis* C101910（C101910）および *Lactococcus lactis* subsp. *lactis* NBRC 12007（NBRC 12007）はRPHを添加した麹汁培地中で良好な増殖を示し，高活性なバクテリオシンを生産した．C101910とNBRC 12007によって生産されたバクテリオシン溶液を培地に対して10％（v/v）の割合で添加した結果，*L. fructivorans* NBRC 13954^T の増殖は顕著に阻害され，その生菌数は，4時間目には検出限界（1.0×10^2 cells/ml）以下まで減少した．また，C102901，C101910およびNBRC 12007によって生産されたバクテリオシン溶液を培地に対して1％（v/v）の割合で添加した結果，*L. hilgardii* NBRC 15886^T とH130株（単離した菌株）の増殖は殺菌的に抑制され，その生菌数は，初期の生菌数と比較して，培養4時間から12時間以内に3オーダー以上減少した．

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