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Characterization of Antimicrobial Substance Producing *Lactococcus* sp. HM58 Isolated from Gastrointestinal Track of Flounder

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A lactic acid bacterium showing antimicrobial activity against fish pathogen was isolated from gastrointestinal tract of flounder for the purpose of use as an aquaculture probiotics. From the analysis of morphological and physiological characteristics, the isolated strain was named as *Lactococcus* sp. HM58. Antimicrobial substance (AMS) from *Lactococcus* sp. HM 58 showed strong growth inhibitory activity against *Streptococcus* sp., which is a fish pathogenic bacterium. AMS was presumed a proteinaceous compound with stability in heat and wide pH range from 2 to 10. It was started to produce in exponential growth phase and was not produced any more in stationary phase. It showed comparatively broad antimicrobial spectrum against most of gram positive bacteria used for this study. About 84% of *Lactococcus* sp. HM58 was able to survive in the artificial gastric juice though it was low to the extent in the artificial bile juice. In the sensitivity test for various antibiotics, this strain was highly sensitive for doxycycline, erythromycin, amoxicillin clavulanic acid and ampicillin.

Key words: Probiotics, Flounder, Gastrointestinal track, Antimicrobial substance, *Lactococcus* sp. HM58

Introduction

It is well known that the indigenous microbiota of humans and animals provide resistance against pathogenic microorganisms. It has also been reported that some lactic acid bacteria isolated from the gastrointestinal (GI) tract of fish have inhibitory effects against bacterial fish pathogens (Joborn et al., 1997). During the past 50 years, numerous trials were conducted with microorganisms known as probiotics in efforts to improve quality of food species, human health and welfare. Appropriate probiotics applications were shown to improve intestinal microbial balance, thus leading to improved food absorption, and reduced pathogenic problems in the GI tract. Several probiotic species including *Lactobacillus* sp., *Saccharomyces* sp., *Bacillus* sp. and mixed cultures of those strains have been used. With some trials, growth promotion was clearly demonstrated in poultry and pigs compared with control groups (Rengpipat S. et al., 1998). Those results were most promising and gave confidence that further improvements in probiotics applications were possible. Lactic acid bacteria are a group of catalase-negative and gram-positive bacteria which have played very important roles in fermentation of foods. Many lactic acid bacteria produce antimicrobial substances like organic acids, hydrogen, diacetyl, carbon dioxide, and bacteriocins. Bacteriocins are proteinaceous compounds with bactericidal activity against strains of the same or closely related to the bacteriocin producing bacterium. These substances are of particular interest as they are proteinaceous and may thus be degraded during

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digestion by human and other animals (Bibek and Mark, 1992; Choi and Park, 1998; Klaenhammer, 1988; Thomas and Kaiser, 1993; Um and Lee, 1996).

Economic damage in aquaculture industry from the infection of fish pathogens has been increased. The amount of use of various antibiotics was simultaneously increased and several kinds of antibiotics resistance stains have been eventually turned up. Therefore, some substitutive way for the overuse of antibiotics should be approached.

In this study, a lactic acid bacterium was isolated from gastrointestinal track of flounder and investigated its characteristics to develop as a probiotic stain that is acid-resistant, bile acid-resistant, and antibiotic-sresistant while repressing the fish pathogenic microbes.

Materials and Methods

Bacterial strains and media

The isolates from GI tract of flounder were used for screening of lactic acid bacteria producing the antimicrobial substance (AMS) and the strains obtained from the Korean Culture Center of Microorganisms (KCCM) were used for determining the antibacterial spectrum. Some of fish pathogens were isolated in our laboratory and identified rapidly using Micro Station System 3.5 (Biolog Co. USA). *Lactococcus lactis* subsp. *lactis* KCCM 40104 was grown in MRS broth (Difco, Detroit, U.S.A) at 30℃. Fish pathogenic bacteria, *Vibrio anguillarum* (PK), *Aeromonas hydrophila* (PK), *Edwardsiella tarda* (PK), *Streptococcus* sp. (PK), *V. carchariae* (isolated and identified for this study; this study), and *E. ictaluri* (this study) were propagated in trypticase soy broth (Difco) added 1% NaCl. *V. parahaemolyticus* (KCCM 11965) was grown in trypticase soy broth (Difco) added 3% NaCl, *Str. agalactiae* (KCCM 11957), *Salmonella enteritidis* (KCCM 12021), *S. choleraesuis* subsp. *choleraesuis* (KCCM 40089), *S. typhimurium* (KCCM 40253), *B. subtilis* (KCCM 11316), *E. faecium* (KCCM 11968), *E. faeculis* (KCCM 11729), *Staphylococcus aureus* (KCTC 1621), and *Escherichia coli* JM109 (KCCM 70082) were propagated in trypticase soy broth (Difco) for determining the antimicrobial activity.

Isolation of lactic acid bacteria from GI track of flounder

Lactic acid bacteria were isolated from GI track of flounder. Homogenized GI track was serially 10-fold diluted with 3% NaCl solution, and plated on MRS medium added 0.01% Bromo cresol purple (BCP) and 3% NaCl. The plates were incubated microaerobically at 25℃ for 3 days and yellow zone forming colonies were isolated.

Screening of AMS producing lactic acid bacteria

For the detection of AMS activity, the paper disk diffusion method was used as described by Zajdak (Zajdak et al., 1985). The isolated strain HM58 was transferred twice in MRS broth before use. After incubation of strain HM58 in MRS broth for 24 hrs at 25℃, the cells were removed by centrifugation (1,500×g, 20 min). For the paper disk diffusion assay, MRS hard agar (containing 2% agar) plates were overlaid with 7 mℓ MRS soft agar (containing 0.7% agar) inoculated with 100 μℓ of the overnight-incubated culture of *Streptococcus* sp. as an indicator strain. 50 μℓ of cell-free supernatant was loaded on the paper disk of overlaid soft agar. After overnight incubation, the strains formed growth inhibitory zone around the disk were selected and maintained as frozen stock cultures at −70℃ in MRS broth with 20% glycerol.

Identification of AMS producing strain HM58

To identify the AMS producing strain HM58, several morphological and cultural characteristics were examined. These test included cell morphology, gram stain, spore formation, motility, growth in MRS broth at different temperatures and pHs and growth in MRS broth supplemented with various concentrations of NaCl. Physiological characteristics were tested by catalase, oxidase and hemolysis raction. Biochemical tests were performed by examination of carbohydrate fermentation patterns using a Micro Station System (Dubois et al., 1979; Gilliand and Speck, 1997).

Determination of AMS activity

The AMS activity was confirmed by the paper disk diffusion method (Zajdak et al., 1985). 50 μℓ of serial two-fold diluted culture supernatant of

strain HM58 with 50 mM sodium phosphate buffer (pH 6.0) was loaded on the paper disk of a plate overlaid with *Streptococcus* sp., and incubated at 30 ℃. The activity was defined as the reciprocal of the highest dilution showing an inhibition zone and was expressed in activity units (AU) per mℓ. *L. lactis* subsp. *lactis* (KCCM 40104) was used to make a comparison between the AMS activity of strain HM58.

Effect of enzymes, heat treatment and pH on AMS activity

The AMS stability were determined in various enzyme solutions, α-amylase (830 U/mg) in 50 mM sodium acetate buffer (pH 6.0), cellulase (8.9 U/mg) in 30 mM sodium acetate buffer (pH 5.0), pepsin (439 U/mg) dissolved in 10 mM citrate buffer (pH 2.0), proteinase K (30 U/mg), protease IX, protease XIV, and trypsin (9,590 U/mg) in 50 mM Tris-HCl buffer (pH 7.5). Cell-free supernatant was dissolved in buffers recommended by the supplier (Sigma Co.) and incubated with each enzyme solution at a final concentration of 1 mg/mℓ for 2 hrs and 24 hrs at 37℃. The reaction mixtures containing α-amylase and trypsin were incubated at 20℃ and 25 ℃, respectively. Untreated AMS and buffer mixture, buffer alone, and enzyme solution were used as controls. The residual AMS activity was determined by the paper disk diffusion method. The effect of heat treatment on AMS activity was examined at 60 ℃, 80℃, 100℃ for 1 min, and autoclave condition (121℃, for 20 min), respectively. Assay for residual activity was performed after cooling down to room temperature. The pH stability of AMS was examined after 4hrs storage at 4℃ in the following buffer: 50 mM glycine-HCl (pH 2.0−4.0), 50 mM citrate (pH 4.0−6.0), 50 mM phosphate (pH 6.0−8.0) and 50 mM Tris-HCl (pH 8.0−10.0).

Production of AMS

Strain HM58 was incubated in MRS broth at 25℃ for 30 hrs. During incubation, the cell growth (OD at 660 nm), AMS activity and pH change were measured to proper intervals.

Antimicrobial spectrum of AMS

The antimicrobial spectrum was tested against several bacteria by a paper disk diffusion method. Indicator strains were previously subcultured in the appropriate medium and then inoculated in the soft agar medium.

Acid and bile tolerance of strain HM58

Acid tolerance was carried out in MRS broth (pH 2.5) added pepsin (1,000 U/mℓ) as artificial gastric juice condition by Kobayashi (Kobayashi et al., 1974) method. Strain HM58 was incubated in MRS broth at 25℃ for 24 hrs. After incubation, cells were harvested by centrifugation (15,000×g for 20 min at 4℃), washed twice in sterile 0.85% NaCl solution, and inoculated (10^8/mℓ cells) into artificial gastric juice. Cells contacted with artificial gastric juice for 30 min at 25℃ spreaded and counted on MRS plates at 10 min intervals. Bile tolerance was performed in MRS broth (pH 4.0) added trypsin (1,000 U/mℓ) (Anonymous, 1986), 0.5% oxgall as artificial bile juice condition. For bile tolerance test, cells incubated in artificial gastric juice for 30 min were used. The cell pellets obtained by centrifugation were resuspended into artificial bile juice. Cells incubated with artificial bile juice for 30 min at 25℃ were counted on MRS plates at 10 min intervals.

Antibiotics sensitivity of strain HM58

To confirm the antibiotics sensitivity, culture broth of strain HM58 was inoculated in 7 mℓ of soft MRS agar, then overlaid onto hard MRS agar. The antibiotic discs containing ampicillin, amoxicillin, clavulanic acid, doxycycline, gentamicin, oxytetracycline, amikacin, ciprofloxacin, erythromycin, ofloxacin and pefloxacin were laid onto soft agar and the growth inhibitory zones against strain HM58 were measured.

Results and Discussion

Screening of AMS producing lactic acid bacteria

Yellow zone forming lactic acid bacteria on MRS agar containing BCP were isolated from the GI track of flounder. These isolates were tested for antimicrobial activity against fish pathogenic bacteria using the paper disk diffusion method. Among them, a strain HM58 (3,200 AU) showed

comparatively higher inhibitory activity against *Streptococcus* sp. than *L. lactis* subsp. *lactis* (160 AU) as a control strain (Fig. 1). Therefore, strain HM58 was selected for further study.

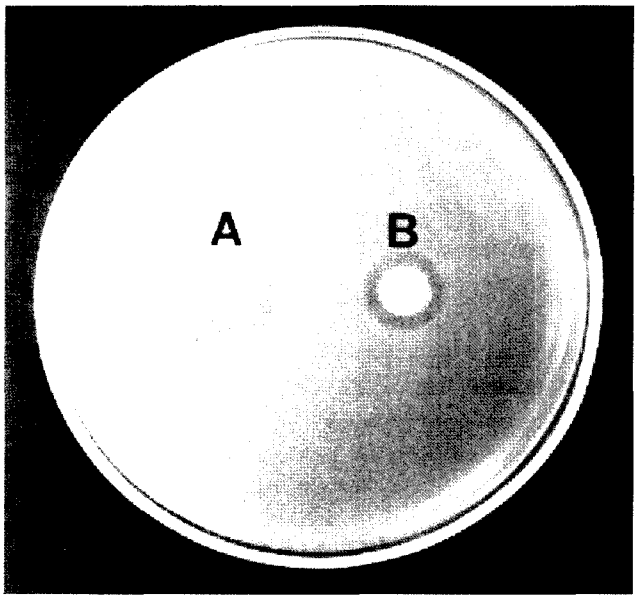

Fig. 1. Antimicrobial activity of isolated strain HM58 against *Streptococcus* sp. A: HM58, B: *Lactococcus lactis* subsp. *lactis* KCCM 40104.

Identification of isolate HM58

The morphological and physiological characteristics of strain HM58 are summarized in Table 1. The strain HM58 was gram-positive, nonmotile, catalase-negative, and coccus (Fig. 2). The carbohydrate utilization patterns (Table 2) by Biolog System coincided with those of *Lactococcus* sp.. Therefore, this strain was classified as *Lactococcus* sp., and named as *Lactococcus* sp. HM58.

Effect of enzymes, heat treatment and pH on AMS activity

The effects of various enzymes on AMS activity are shown in Table 3. The AMS activity was lost by treatment some enzymes such as proteinase K, protease IX, protease XIV and pepsin, suggesting that the AMS from *Lactococcus* sp. HM58 was a proteinaceous compound in nature. However, it was not inactivated in the presence of trypsin, cellulase, α-amylase and cellulase, respectively. The activity was unaffected by heat treatment up to 100℃ for 1 min but completely inactivated at autoclave condition (data not shown). The AMS activity was

Table 1. Morphological and physiological characteristics of isolated strain HM58

Classification	Strain HM58
Morphology characteristics	
Shape	cocci or egg shape
Colony	circular / convex
Gram stain	+
Spore	−
Motility	−
Culture characteristics	
Growth in air	+
Growth at anaerobic condition	+
Growth at 10℃	+
45℃	+
Growth in broth at pH 4.5	+
pH 9.6	+
Growth in broth with 3% NaCl	+
4% NaCl	+
6% NaCl	−
15% NaCl	−
Physiological characteristics	
Catalase reaction	−
Oxidase reaction	−
Litmus milk reaction	
Acid formation	+
Reduction	+
Curd formation	+
Gas from glucose	−
Hemolysis	−
Cellulose hydrolysis	−
Protein hydrolysis	+
Starch hydrolysis	−

+, positive reaction; −, negative reaction.

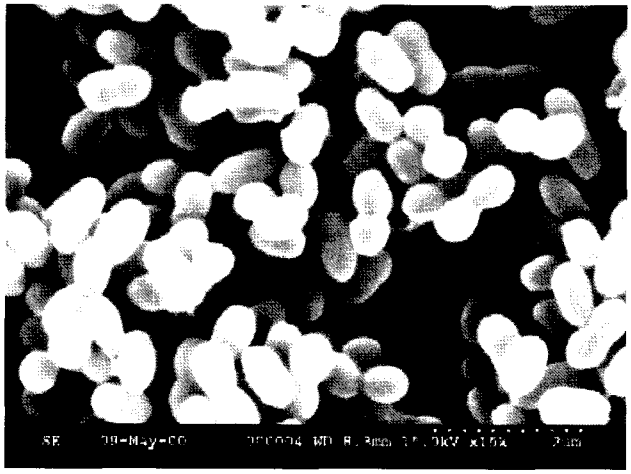

Fig. 2. Scanning electron micrograph (SEM) of the isolated strain HM58.

Table 2. Results of carbohydrate utilization of isolated strains

Carbohydrate	HM58	Carbohydrate	HM58
α-cyclodextrin	+	Maltotriose	+
β-cyclodextrin	+	Mannitol	−
Dextrin	−	D-mannose	−
Tween 40	−	D-melezitose	−
N-acetyl glucosamine	+	β-methyl D-galactoside	−
Amygdalin	−	β-methyl D-glucoside	+
L-arabinose	+	D-ribose	−
Arbutin	+	Salicin	+
Cellulose	+	Sucrose	+
D-fructose	+	D-trehalose	+
D-galactose	+	L-lactic acid	−
Gentiobiose	+	Methyl pyruvate	−
α-D-glucose	+	Glycerol	−
α-D-lactose	+	Adenosine	−
Lactulose	+	Inosine	−
Maltose	+	Uridine	−

Table 3. Effect of various enzymes treatment on the AMS activity

Enzyme treatment	Residual activity (AU/mℓ)	
	2 hrs	24 hrs
Control		
AMS+Buffer	320	320
Buffer	0	0
Enzyme solution	0	0
α-Amylase (830 U/mg)	320	320
Cellulase (8.9 U/mg)	320	320
Pepsin (859 U/mg)	320	0
Protease IX	320	80
Protease XIV	0	0
Proteinase K (30 U/mg)	320	80
Trypsin (10,200 U/mg)	320	320

Cell-free supernatant was treated with 1 mg/mℓ of enzyme solution and incubation at optimal temperature.

maintained stably between pH 2.0 and pH 10.0 at 4℃ (data not shown).

Production of the AMS

The AMS activity by *Lactococcus* sp. HM58 was detected at the early exponential growth phase and reached the maximum at the stationary phase (Fig. 3). According to the results of Ahn et al. (Ahn, 1993; Ahn and Stiles, 1990), bacteriocin has been produced at the late-exponential or stationary phase as a typical secondary metabolite. So, it could be considered that the AMS activity is different with those from lactic acid bacteria.

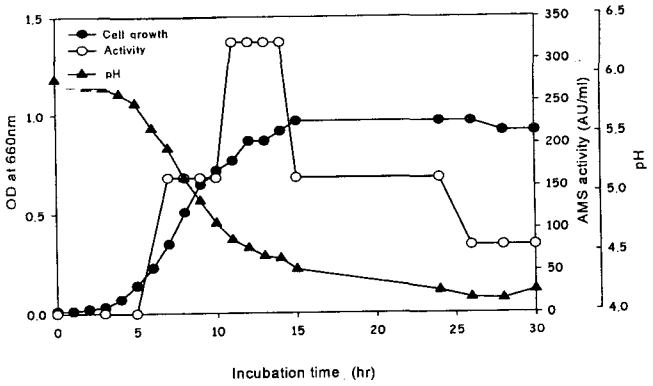

Fig. 3. Cell growth, AMS production and pH variation of *Lactococcus* sp. HM58 in MRS medium at 25℃. AMS activity was measured by dilution analysis on *Streptococcus* sp. indicator agar plate.

Antimicrobial spectrum of the AMS

The antimicrobial spectrum of the AMS was tested against various gram-negative and positive bacteria. The results are shown in Table 4. It showed antimicrobial spectrum against most of gram positive bacteria except *S. aureus*. The AMS was not shown any activity against gram-negative bacteria used for this study.

Acid and bile tolerance of *Lactococcus* sp. HM 58

The broth of *Lactococcus* sp. HM58 cultured overnight was inoculated into artificial gastric juice added 1,000 U/mℓ pepsin with 10^8/mℓ. The cells were counted at 10 min intervals for 30 min (Table 5). *Lactococcus* sp. HM58 was survived at the level of 91% after 10 min, to 88% after 20 min and to 82% after 30 min. The pure gastric juice has pH between 1.4 and 2.0, which is not survivable to most of microbes. But with ingesting of nutrition, the pH of animal gastric juice will be increased to prevent microbes from dying. To demonstrate the physiological function of probiotic candidates in fish body, it should be able to survive in the stomach. The survival capability of *Lactococcus* sp. HM58 was reduced with the lapse of time. But fishes used to discharge their waste within 90 min after feed. Therefore *Lactococcus* sp. HM58 can be tolerated enough in fish stomach condition. Table 5 showed the number of alive bacteria survived in artificial gastric juice and artificial bile juice. Comparatively low survival rate in artificial bile juice might be

Table 4. Antimicrobial spectra of *Lactococcus* sp. HM58

Test organisms	HM58
Gram positive bacteria	
Bacillus subtilis (KCCM 40089)	++
Enterococcus faecium (KCCM 11968)	++
Enterococcus faeculis (KCCM 11729)	+++
Staphylococcus aureus (KCTC 1621)	−
Streptococcus agalactiae (KCCM 11957)	+++
Streptococcus sp. (PK)	+++
Gram negative bacteria	
Escherichia coli JM109 (KCCM 70082)	−
Aeromonas hydrophila (PK)	−
Edwardsiella tarda (PK)	−
Edwardsiella ictaluri (this study)	−
Salmonella choleraeusis subsp. *choleraesuis* (KCCM 40089)	−
Salmonella enteritidis (KCCM 12021)	−
Salmonella typhimurium (KCCM 40253)	+
Vibrio parahaemolyticus (KCCM 11965)	−
Vibrio anguillarum (PK)	−
Vibrio carchariae (this study)	−

*PK, strains donated by department of aquatic life medicine Pukyung National University; −, No inhibition zone; ++, Diameter of inhibition zone 10 to 12 mm; +++, >12 mm.

Table 5. Survival of *Lactococcus* sp. HM58 in artificial gastric juice and bile juice for 30 min

Treat (30 min)	Artificial gastric juice[1]		Artificial bile juice[2]	
Cell No.	Control[3]	Treat	Control[4]	Treat
CFU/mℓ	2.2×10^8	1.8×10^8	1.8×10^8	1.4×10^8
survival rate (%)	82 ± 0.5		78 ± 0.5	

1, Broth containing 1,000 U/mℓ of pepsin (pH 3.5, adjusted with 1 N HCl); 2, Broth containing 0.5% oxgall, 1,000 U/mℓ trypsin (pH 7.5, adjusted by NaOH); 3, Cell number of *Lactococcus* sp. in MRS broth containing fructose as carbon source; 4, Cell number after treatment by artificial gastric juice for 30 min.

from artificial bile condition of livestock. From these results, we supposed that *Lactococcus* sp. HM 58 can be actively survive in fish stomach condition during ingestion of feed.

Antibiotics sensitivity of *Lactococcus* HM58

Sensitivities of *Lactococcus* sp. HM58 against 12 different antibiotics that are widely used in aquaculture have been checked. The result showed that the strain was highly sensitive against doxycycline, erythromycin, amoxicillin clavulanic acid, and ampicillin (Table 6). These characteristics could be useful basic information when the strain might be of use in parellel with antibiotics selected in aquaculture.

Table 6. Antibiotic sensitivity test of *Lactococcus* sp. HM58

Antibiotics	Diameter (mm)
Ciprofloxacin (5 mcg)	0
Gentamicin (10 mcg)	0
Doxycycline (30 mcg)	20
Erythromycin (15 mcg)	16
Ofloxacin (5 mcg)	<1
Amoxycillin clavulanic acid (30 mcg)	23
Ampicillin (10 mcg)	20
Streptomycin (10 mcg)	0
Amikacin (30 mcg)	0
Oxolinic acid (2 mcg)	0
Neomycin (5 mcg)	0

Antibiotic sensitivity tested by dispens-o-disc (Difco co.).

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