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Bacteriocin with a Broad Antimicrobial Spectrum, Produced by *Bacillus* sp. Isolated from Kimchi

MAH, JAE-HYUNG, KWANG-SOO KIM, JONG-HYUN PARK[1], MYUNG-WOO BYUN[2], YOUNG-BAE KIM, AND HAN-JOON HWANG*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[1]Department of Food and Bioengineering, Kyungwon University, Sungnam 461-701, Korea
[2]Team for Radiation Food Science and Biotechnology, Korea Atomic Energy Research Institute, Taejon 305-353, Korea

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Abstract An antimicrobially active bacterium which was identified as *Bacillus brevis*, was isolated from kimchi. The antimicrobial activity was found against various Gram-positive and Gram-negative bacteria including some pathogens food-spoilage microorganisms, and some yeast strains. The antimicrobial activity was especially strong against *Bacillus anthracis* and *Shigella dysenteriae*. The strong activity was observed during an early stationary phase in the culture when incubated at 37°C with initial medium pH of 6.8. The antimicrobial activity was found to be stable at 90°C for 30 min and in the pH range of 3–11, and it was insensitive to organic solvents including acetone, acetonitrile, ethanol, and methanol. Analysis of the bacteriocin on tricine-sodium dodecyl sulfate-polyacrylamide gel suggested a molecular mass of approximately 4.5–6.0 kDa. The antimicrobial substance was characterized as a bacteriocin, because of its proteinaceous nature and low molecular weight. The bacteriocin could potentially be used as a food preservative, because of its thermostable property and broad antimicrobial spectrum.

Key words: Bacteriocin, antimicrobial activity, kimchi, *Bacillus brevis*, tricine-SDS-PAGE

Bacteriocins from various bacteria are known as proteinaceous compounds with a bactericidal mode of action against closely related organisms. Therefore, their activities are considered to be species-specific [36]. Under normal conditions, Gram-negative bacteria seem to be insensitive to bacteriocins, since the activity against Gram-negative bacteria is rarely observed [17, 40]. Lactic acid bacterial (LAB) bacteriocins are generally active against only Gram-positive species, which are not inhibitory toward Gram-negative bacteria [7, 28]. Recent studies are limited to LAB bacteriocins and their activity against *Listeria monocytogenes* [7]. Of LAB bacteriocins, brevicin [31] and carnocin [37] are resistant to heat treatment up to 100°C, and they have narrow antimicrobial spectra.

The genus *Bacillus* includes a variety of industrially important species, such as enzymes, antibiotics, amino acids, and insecticides. Similar to lactic acid bacteria, some *Bacillus* strains such as *B. subtilis* and *B. licheniformis* are "generally recognized as safe" (GRAS) bacteria [35]. Within the genus *Bacillus*, bacteriocins or bacteriocin-like substances have been reported to be found in *B. subtilis* [18], *B. megaterium* [16], *B. stearothermophilus* [34], *B. licheniformis* [5], *B. thuringiensis* [8], *B. cereus* [27, 30], and *B. coagulans* [15]. The best characterized bacteriocins are subtilin [2, 11, 18, 21, 22, 29] of the *B. subtilis* and megacin [38, 39] of *B. megaterium*. Among the *Bacillus* bacteriocins, a bacteriocin was found to be heat labile and not broadly effective for the antimicrobial activity, although both cerein [27] and coagulin [15] are not affected by several organic solvents. Kimchi is a Korean traditional fermented vegetable product that is a good source to screen new bacteriocin producers [19, 23]. In the present study, we investigated the antimicrobial activity of the bacteria isolated from kimchi and the characterization of a strongly antagonistic substance.

Materials and Methods

Strains and Media

The strain which had been isolated from kimchi [25] was tested for antimicrobial activity. Indicator organisms for screening antimicrobial activities were obtained from the

*Corresponding author
Phone: 82-2-923-7769; Fax: 82-2-3290-3437;
E-mail: hjhwang@korea.ac.kr

Korean Collection for Type Culture (KCTC) and American Type Culture Collection (ATCC). They were grown in nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.) at 37°C and Yeast Malt Extract (YM) broth (Difco) at 30°C. Hard agar [24] containing glucose 40 g/l, yeast extract 20 g/l, and agar 20 g/l plates was used for the agar diffusion test.

Identification of Strain with Antimicrobial Activity

Morphological, physiological, and cultural properties were examined to identify the strain with antimicrobial activity.

Table 1. Morphological and biochemical characteristics of isolate No. 430.

Characteristics	Isolate No. 430
Shape	rod
Gram stain	+
Spore stain	+
Catalase	+
Oxidase	-
Anaerobic growth	+
Motility	(+)
Swimming	(+)
Twitching	+
Voges-Proskauer test	-
pH in V-P broth	(6.50)
<6	-
>7	-
Acid from	
D-Glucose	+
L-Arabinose	-
D-Xylose	(+)
D-Mannitol	-
Gas from glucose	-
Hydrolysis of starch	+
Utilization of citrate	-
Formation of indole	-
NaCl and KCl required	-
Allantoin or urate required	-
Growth at pH	
6.8, nutrient broth	+
5.7	+
Growth in NaCl	
2%	+
5%	+
7%	(+)
10%	-
Growth at	
5°C	-
10°C	-
30°C	+
40°C	+
50°C	+
55°C	+
65°C	-

These tests including cell morphology, Gram stain, spore formation, motility, biochemical test, growth in a nutrient broth at different temperatures and pH, and growth in a nutrient broth supplemented with various concentrations of NaCl (shown in Table 1) were performed according to Bergey's Manual [6, 12].

Fatty acid methyl ester was prepared and extracted by the standard protocol of the Microbial Identification System (MIDI: Microbial ID, Inc., Newark, DE, U.S.A.). The extract was analyzed by a Hewlett-Packard model HP 6890 gas chromatograph, equipped with a 25 m×0.2 mm methyl phenyl silicone fused silica capillary column (HP 19091B-102).

Detection of Antimicrobial Activity

Antimicrobial preparation was made from a cell-free supernatant of MRS broth (Difco) culture [4]. After cultivation for 18 h, centrifugation at 3,500 ×g for 10 min followed. The supernatant was concentrated 10-fold by using a vacuum evaporator (Model N-1NW, EYELA Co., Tokyo, Japan). The concentrated liquid was adjusted to pH 6.8 usinga 10 M NaOH solution and filter-sterilized by passing through a 0.45 μm filter (Millipore Co., Bedford, MA, U.S.A.). Filter-sterilized catalase from bovine liver (Sigma Chemical Co., St. Louis, MO, U.S.A.) in a 50 mM phosphate buffer was added at a final concentration of 100 U/ml.

The antimicrobial activity was determined using the spot-on-lawn method [13], the cross-streaking method [13], along with the agar diffusion test [24].

Influence of Temperature and pH of the Medium on Production of the Antimicrobial Substance

The optimum temperature and pH of the medium for the production of antimicrobial substance were determined at a range of 30–38°C and initial pH level of 5.0–7.0. To investigate the relationship between growth and antimicrobial production of the strain, the culture was tested at different time intervals by applying the absorbance measurement (A_{600}) (DU 650 spectrophotometer, Beckman, U.S.A.).

Effects of Different Treatment on Antimicrobial Activity

The effects of heat, pH, organic solvents, and proteolytic enzymes on antimicrobial activity were investigated. To investigate the effect of heat on bacteriocin activity, antimicrobial preparation was heated at 45, 60, 75, and 90°C for 15 and 30 min, and then immediately cooled in ice water. The pH stability was examined with the antimicrobial preparation with the pH adjusted to levels between pH 3.0 and 11.0, and then incubated at room temperature for 1 h. Each was readjusted to neutrality, and the volumes equalized. Various organic solvents including acetone, acetonitrile, ethyl alcohol, and methyl alcohol at the final concentration of 10% were added to the antimicrobial

preparation, and then incubated at 25°C for 1 h. The antimicrobial preparation was also treated with a final concentration of 1 mg/ml proteolytic enzymes (Sigma) in 50 mM phosphate buffer (pH 7.0) at 37°C for 1 h: α-chymotrypsin, proteinase K, and pronase E. Added organic solvents or proteolytic enzymes had the same volume as the antimicrobial preparation, and then a 2-fold-diluted antimicrobial preparation (5-fold-concentrates of cultures) was used for detecting the residual antimicrobial activity. The volumes were not equalized due to volatilization of organic solvents or inactivation of proteolytic enzymes.

Partial Purification of Antimicrobial Substance for SDS-PAGE

The culture of isolate was collected 30–36 h after the incubation. The culture was centrifuged at 11,000 ×g at 4°C for 15 min, adjusted to pH 7.0, filter-sterilized (0.45 μm filter), heated for inactivation of proteinase, and precipitated with 75% ammonium sulfate. Slow stirring was continued at 4°C for an additional 2 h. Precipitated protein was pelleted by centrifugation at 11,000 ×g at 4°C for 15 min. The pellet was resuspended in 10 mM phosphate buffer (pH 7.0). After removing the ammonium sulfate by dialysis with 10 mM phosphate buffer (pH 7.0) (dialysis membrane Mw cut-off: 1,000 Da) (Spectrum Medical Industries, Inc., CA, U.S.A.), the dialyzed sample was sterilized by passing through a 0.45 μm filter.

Direct Detection of Antimicrobial Activity on Tricine-SDS-PAGE

To estimate the molecular weight of the antimicrobial substance, a tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% urea (Tricine-SDS-PAGE with 10% Urea) [33] was performed. At the end of electrophoresis, the two gels were removed. One gel containing the antimicrobial substance and molecular weight standards was stained with Coomassie brilliant blue R250 (Sigma). The other gel containing only the antimicrobial substance was tested for antimicrobial activity [3]. The gel was immediately fixed by treatment for 3 h in 50% methanol, 10% acetic acid in distilled water, and washed for 1.5 h in distilled water. The gel was then placed into a sterile petri dish and overlaid with 10 ml of nutrient soft agar containing 1 ml of an overnight culture of *Bacillus subtilis* KCTC 1021. Then, the dish was incubated at 30°C for about 12–24 h, and an inhibition halo was observed during this time period.

RESULTS

Isolation and Identification of the Strain Producing an Antimicrobial Substance

Of 120 strains which were isolated from kimchi, 44 strains showed some antimicrobial activity against various indicator organisms. Among the 44 strains, isolate No. 430 was finally selected, because it not only showed the highest antimicrobial activity among all the strains tested, but it was also insensitive to catalase.

Table 2. Composition of fatty acids in the strain with antimicrobial activity isolated from kimchi.

Fatty acid	Content (%) in isolate No. 430
14:0 ISO	7.91
15:0 ISO	15.02
15:0 ANTEISO	54.06
16:0 ISO	13.96
16:1 w11c	–
16:0	–
ISO 17:1 w10c	–
17:0 ISO	–
17:0 ANTEISO	9.05
Identified result	*B. brevis*
SI	0.238

Various properties of the isolate are shown in Tables 1 and 2 and Fig. 1. Isolate No. 430 was identified as an endospore-forming, Gram-positive, catalase-positive, and motile rod. These characteristics suggested the genus *Bacillus*. The pattern of fatty acid profile by the MIDI system suggested the species to be *B. brevis*, even though the similarity was not enough to support the result (SI=0.238). The strain was identified as *B. brevis*, because of the most likely morphological and biochemical properties defined by Bergey's Manual [6, 12].

Antimicrobial Spectrum of the Isolate

The inhibitory spectrum of the isolate No. 430 is shown in Table 3. The isolate showed antimicrobial activity against not only various Gram-positive bacteria, but Gram-negative ones as well. It also showed a very strong activity against *B. anthracis* and *Shigella dysenteriae*. Furthermore, yeast strains such as *Pichia anomala* and *Saccharomyces cerevisiae* were also inhibited, thus showing a very broad spectrum of inhibition ranging from prokaryotes to some eukaryotes.

Influence of Temperature and Initial pH of the Medium on the Production of the Antimicrobial Substance

As shown in Fig. 2, the optimum incubation temperature of the strain was observed at 37°C. Its highest antimicrobial activity was detected at an initial pH 6.8 of the medium (Fig. 3). When the temperature during the incubation process was below 34°C or an initial pH of the medium was below 6.5, the growth of the isolate was delayed (data not shown) and the antimicrobial activity was decreased as well. The amount of antimicrobial product in a medium was dependent upon the phase of bacterial growth (Fig. 4). No activity was detected during the first 15 h of

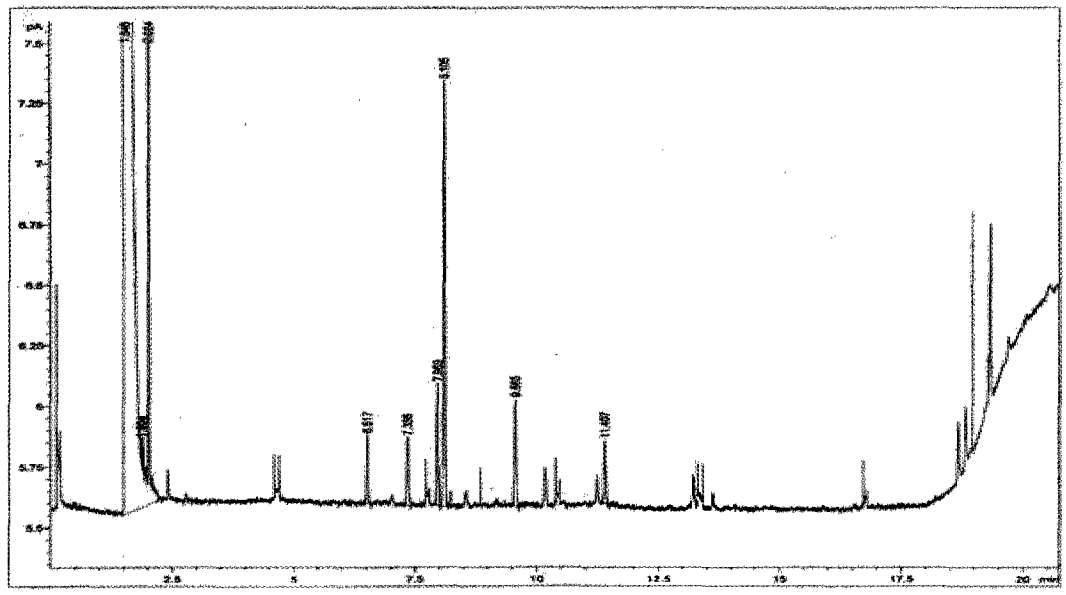

Fig. 1. Chromatographic profile of the fatty acid composition of isolate No. 430.

incubation. The antimicrobial activity of the isolate was observed after the mid-log phase, and it reached its maximum level at the early stationary phase and eventually decreased after the late stationary phase.

Table 3. Inhibitory spectrum of the antimicrobial substance produced by isolate No. 430.

Indicator	Antimicrobial activity in agar diffusion test
Gram-positive	
Bacillus anthracis ATCC 14185	++++[1)]
Bacillus cereus KCTC 1012	+++
Bacillus subtilis KCTC 1021	++
Enterococcus faecium KCTC 2022	+
Listeria monocytogenes ATCC 19113	+
Staphylococcus aureus KCTC 1916	+
Gram-negative	
Escherichia coli KCTC 1039	+
Escherichia coli O157:H7 ATCC 43894	+
Enterobacter aerogenes KCTC 2190	++
Salmonella typhimurium KCTC 1925	+
Pseudomonas aeruginosa KCTC 1750	+
Pseudomonas fluorescens KCTC 2344	++
Shigella dysenteriae ATCC 13313	++++
Yersinia enterocolitica ATCC 23715	++
Yeasts	
Pichia anomala	+
Saccharomyces cerevisiae	+

[1)]+, ++, +++, ++++: Relative activity in comparison with activity of control.
+ indicates control diameter+up to 2 mm.
++ indicates control diameter+up to 4 mm.
+++ indicates control diameter+up to 6 mm.
++++ indicates control diameter+over 6 mm.
Antimicrobial preparation: concentrated 10-fold from cell-free supernatant adjusted to pH 6.8, and treated with catalase

Effect of Different Treatments on Antimicrobial Activity

As shown in Table 4, the antimicrobial substance was stable under 60°C and the activity still remained after 30 min at 90°C. The highest activity was detected at pH 7 and the residual activity was still detected in the range of pH 3–11. The antimicrobial activity was not affected by treatments with organic solvents. However, the antimicrobial activity was completely lost by treatments with proteinase K and α-chymotrypsin, and partially inactivated by pronase E. This indicated that the antimicrobial substance produced by isolate No. 430 had a proteinaceous nature that could be classified as a bacteriocin (Fig. 5).

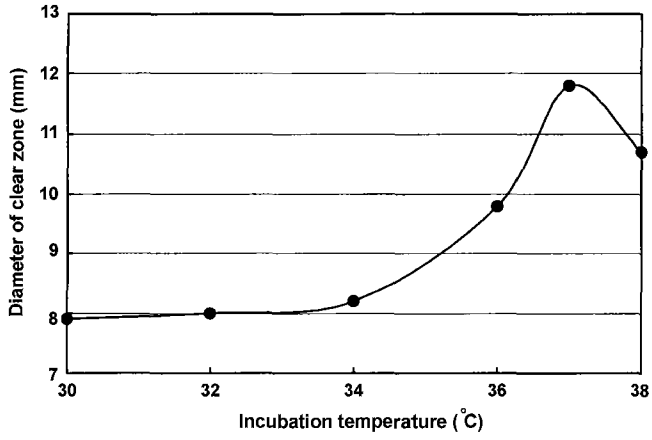

Fig. 2. Effect of incubation temperature on the antimicrobial activity of isolate No. 430.

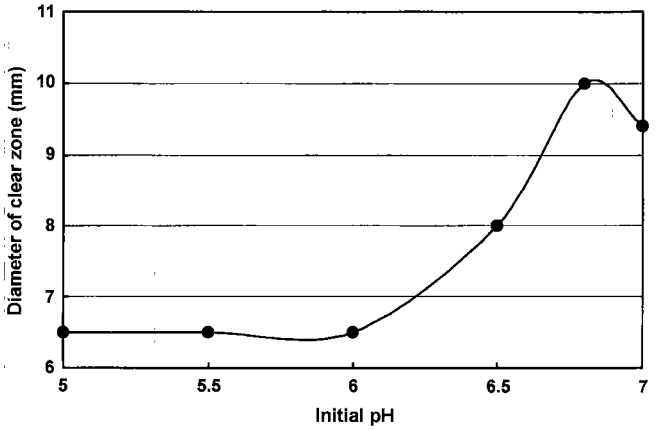

Fig. 3. Effect of the initial pH of the culture medium on the antimicrobial activity of isolate No. 430 at 37°C.

Tricine-SDS-PAGE with 10% Urea

The electrophoretogram of the gel showed several protein bands of bacteriocin that were produced by isolate No. 430, as measured against a protein molecular weight standard ranging approximately from 6 to 200 kDa (Fig. 6).

As shown in Fig. 6, the other gel showed an inhibitory zone that was spread at the position of 4.5–6.0 kDa of the isolate, but an inhibitory zone of the control was not observed. The zone of bacteriocin activity was not found to be associated with any specific protein band stained with Coomassie brilliant blue.

DISCUSSION

Most of the bacteriocins show a narrow spectrum of antimicrobial action; they inhibit strains closely related to the producers. Only a few bacteriocins from Gram-positive

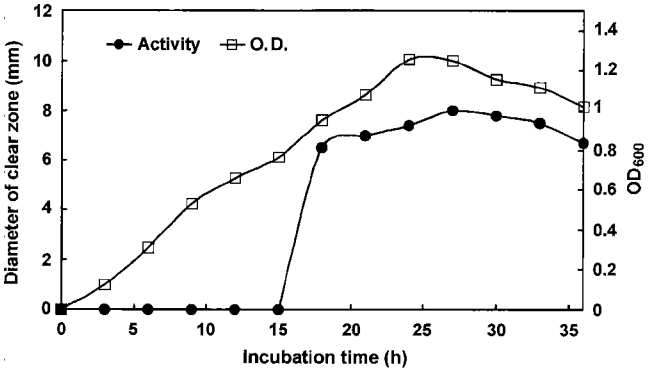

Fig. 4. Correlation between growth and antimicrobial activity of isolate No. 430.

Open symbol (□) refers to the growth curve of the strain in MRS broth at 37°C. Closed symbol (●) indicates the diameter of the clear zone observed by agar diffusion test with antimicrobial preparation harvested at different times during the incubation process.

Table 4. Effect of different temperature, pH, and organic solvents on the stability of the antimicrobial substance.

Treatment	Antimicrobial activity of isolate No. 430 in agar diffusion test		
	B. subtilis	*E. coli*	*E. coli* O157:H7
Control[a]	10.1[1)]	7.2	7.2
Heat treatment			
60°C, 15 min	10.0	6.7	6.8
75°C, 15 min	10.0	6.5	6.7
90°C, 15 min	10.2	–	6.5
60°C, 30 min	10.0	6.5	6.6
75°C, 30 min	10.0	–	6.5
90°C, 30 min	10.2	–	6.5
pH treatment			
pH 3	9.2	–	–
pH 5	9.6	6.7	–
pH 7	10.1	7.0	–
pH 9	9.6	–	–
pH 11	8.0	(+)	–
Control[b]	9.3	–	–
Organic solvents			
Acetone	9.8	–	–
Acetonitrile	9.4	–	–
Ethyl alcohol	9.5	–	–
Methyl alcohol	9.2	–	–

[1)]diameter (mm) of clear zone included disk (ϕ 6 mm).
–: no inhibition zone.
(+): the level of antimicrobial activity corresponding to control.
Antimicrobial preparation: concentrated 10-fold from cell-free supernatant adjusted to pH 6.8, and treated with catalase.
[a]Control was made from MRS broth that was treated by the same procedure as the antimicrobial preparation.
[b]Control was made from MRS broth that was treated by the same procedure as the antimicrobial preparation, and then 2-fold-diluted by adding each organic solvent.

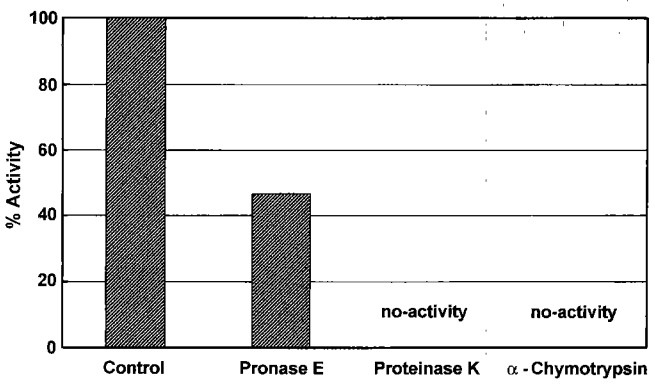

Fig. 5. Inactivation of the antimicrobial substance by various proteolytic enzymes.

The antimicrobial preparation was treated for 1 h at 37°C with various enzymes at a final concentration of 1 mg/ml before being tested for antimicrobial activity against *Bacillus subtilis*. All proteolytic enzymes were dissolved in 50 mM of phosphate buffer (pH 7.0). An enzyme-untreated antimicrobial preparation in the phosphate buffer alone served as a control.

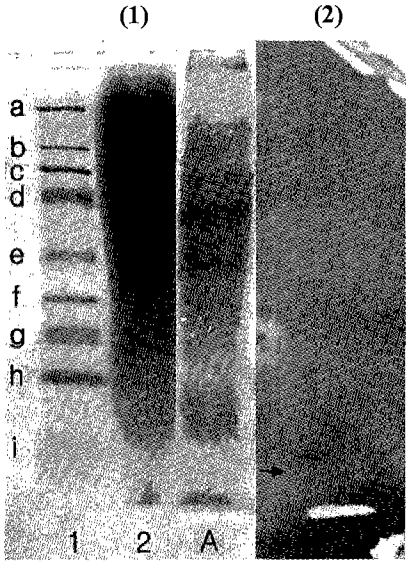

Fig. 6. Tricine-SDS-PAGE with 10% urea, of the cell-free supernatant of culture of isolate No. 430 (lane A).
(1) is the stained half of the Tricine-SDS-PAGE gel. Lane 1 of (1) contains the following protein markers: a, 200; b, 116; c, 97.4; d, 66; e, 45; f, 31; g, 21.5; h, 14.5; i, 6 kDa. Lane 2 is the control. (2) shows direct detection of antimicrobial activity. The gel was overlaid with the soft agar containing *Bacillus subtilis* to estimate the molecular weight of the antimicrobial fraction. The arrow indicates the inhibition halo that was observed after overnight incubation at 37°C.

bacteria inhibit diverse groups of Gram-positive bacteria, but fewer inhibit Gram-negative bacteria [9, 20, 40]. The antimicrobial substance of isolate No. 430 differed from other bacteriocins because it had a broad antimicrobial spectrum on Gram-negative bacteria as well as Gram-positive bacteria. The preparation was not highly purified, although it was prepared from cell-free supernatant that was neutralized and treated with catalase to remove organic acids and hydrogen peroxide. Therefore, it should be investigated whether or not the purified bacteriocin had the same activity as the partially purified bacteriocin preparation. Isolate No. 430 was identified as *B. brevis* on the basis of physiological, cultural, and biochemical characteristics. In order to complete the identification, the genetic approach such as DNA hybridization and the pattern of electrophoresis are required.

The high stability of antimicrobial substance from isolate No. 430 at various temperatures, pHs, and organic solvents are advantageous. The maximal solubility and stability of nisin used for food preservation are at pH 2 and these parameters decrease significantly as the pH increases, which is a considerable disadvantage for use as an additive in nonacidic foods [11]. Like nisin, bacteriocins and bacteriocin-like substances which are produced by lactic acid bacteria are only stable at acidic and/or neutral condition and they are inactivated at a pH above 8.0 [9]. Considering these points, the antimicrobial substance from isolate No. 430 has an interesting potential for the preservation of nonacidic fermented foods such as Doenjang (Korean soybean paste) that is fermented mainly by *B. subtilis* and *B. natto*, etc. The use of the strain as a starter culture could increase the shelf-life of Doenjang without changing its flavor, since the strain has a broad antimicrobial spectrum. Therefore, it should be investigated whether or not the strain could fulfill the requirements for kinetics, proteolytic activity, and flavor profile as a starter culture during the Doenjang preparation.

There have been a few reports on enhancers for the bacteriocin production, including yeast extract for *Streptococcus mutans* [32] and staphylococci [26], manganese salts for *B. megaterium* [1], and Tween 80 for *B. coagulans* [15] and *Lactococcus lactis* subsp. *cremoris* F46 [14]. MRS medium includes these enhancers. Even though the isolate No. 430 was not a lactic acid bacteria, the culture in MRS broth showed antimicrobial activity greater than the culture grown in a nutrient broth, which may be associated with components of the MRS medium as enhancers.

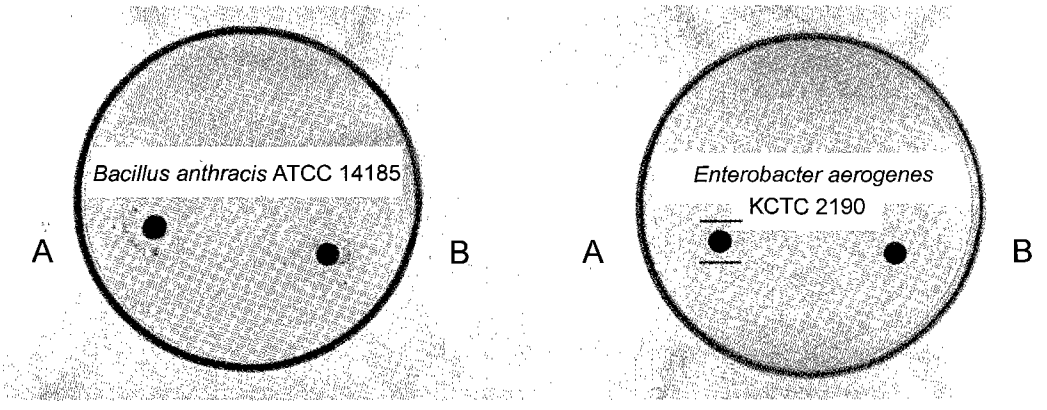

Fig. 7. Antimicrobial effect of isolate No. 430 against *Bacillus anthracis* and *Enterobacter aerogenes*.
The bar (right picture) indicates the diameter of the inhibition halo. Disks A and B were injected by preparation from isolate No. 430 and medium alone, respectively. Each disk (ϕ 6 mm) contained 50 μl of cell-free supernatant that was concentrated 10-fold, adjusted to pH 6.8, and treated with catalase.

The antimicrobial activity of the isolate was inactivated by proteolytic enzymes. This indicated that the antimicrobial substance has a proteinaceous property that can be classified as a bacteriocin. For further studies, there is a need to identify the bacteriocin from isolate No. 430. Douglas *et al.* [10] found that the bacteriocins with low molecular weight below 10 kDa were difficult to be measured by SDS-PAGE, because of an insufficient resolving power of this electrophoresis. On the other hand, our study with tricine-SDS-PAGE with 10% urea was applicable to detect the bacteriocin with low molecular weight.

Acknowledgment

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