Isolation of Lactic Acid Bacteria with Anti-MRSA Bacteriocin Activity and Characterization of the Bacteriocin Product

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This study aimed at isolating lactic acid bacteria with anti-MRSA (methicillin-resistant *Staphylococcus aureus*) bacteriocin activity from fermented shrimp. We selected three strains, named *Weissella* sp. S1, S2, and S3, using analysis based on 16S rRNA gene sequences. All strains showed appropriate growth in an MRS medium containing 5% (w/v) NaCl and showed antibacterial activities against *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and MRSA. The strains exhibited similar growth rates at 0–5% NaCl, with approximate reduction in growth rate observed at 9% NaCl. *Weissella* sp. S1, S2, and S3 exhibited maximum growth rates at pH 7, 9, and 8, respectively. The crude bacteriocin was prepared from *Weissella* sp. S3 and subjected to characterization. The remaining activities after 30 min of exposure at each temperature were 100%, beyond 75%, and 49% at 4°C and 37°C, 50°C and 70°C, and 100°C, respectively. The remaining activities after 24 h of exposure at each pH were 100%, 75%, and 49% at pH 3 and 5, 7 and 9, and 10, respectively. Use of 50% (v/v) ethanol or isopropanol treatment did not diminish the antibacterial activity of the bacteriocin, while the 50% (v/v) hexane treatment reduced the activity by 51%. The molecular weight of the bacteriocin was nearly 6 kDa that was quantified using tricine-SDS-PAGE. Our findings suggest that *Weissella* sp. S3 may be considered a probiotic and useful source of antimicrobial substances in the development of bio-preservatives for food or in MRSA treatment.

Keywords: Antibacterial activity, bacteriocin, fermented shrimp, MRSA, *Weissella* sp.

Introduction

Staphylococcus aureus is a Gram-positive spherical bacterium that is commonly found on the skin and in the noses of healthy people. *S. aureus* is the bacterium that most commonly causes skin disease and food poisoning [1]. However, as a result of the abuse and overuse of antibiotics to treat diseases caused by *S. aureus*, a bacterium resistant to an entire group of antibiotics, including methicillin, has appeared; this bacterium is

known as methicillin resistant *S. aureus* (MRSA) [1, 2]. When MRSA was first reported, it seemed unlikely to become widespread; however, now it has spread all over the world [1, 2]. MRSA is difficult to treat because of general antibiotic resistance as well as due to the fact that it spreads easily between patients within a medical institution. MRSA has been discovered as a form of community infection in adults and children [1, 3]. Research on the development of therapeutic agents for MRSA has mainly focused on the development of new antimicrobial substances and the combined administration of existing antimicrobial agents; however, this has not yet been successful. Therefore, it can be said that there is an urgent need to develop an antibacterial substance with anti-

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icrobial activity against MRSA.

Bacteriocins are antibacterial peptides/proteins that kill the closely related bacteria or inhibit the growth of closely related bacteria. Antibiotics are usually secondary metabolite and can be administered to humans and accumulate in the body [4], causing side effects. Bacteriocins are primary metabolite and cause no bioaccumulation. Heat stability and safety are essential to the use of bacteriocins as food preservation agents. Bacteriocins do not form tertiary structures and have a low molecular weight [5, 6], making them stable to heat [7]. As bacteriocins are natural proteins, they are easily decomposed by proteinases after ingestion, hence they could be safe [8, 9].

Many lactic acid bacteria, which are known to have antibacterial activity against pathogenic bacteria, produce bacteriocins [10] and could be a member of probiotics. Koreans have traditionally fermented salted fish [12]; lactic acid bacteria can easily be found in such fermented seafood [11]. Lactic acid bacteria exhibit various functions such as stabilization of intestinal microbial flora, hyperplasia syndrome, lactose intolerance, remission of non-specific immune specifications and vitamin production [13].

Therefore, it is worth screening for bacteriocin-producing lactic acid bacteria that can be probiotics, and is useful in the development of a safe bio-preservative or MRSA therapeutics. In this study, we isolated three lactic acid bacteria showing antimicrobial activity against MRSA from Korean traditional fermented seafood and the bacteriocin of one prominent strain was purified and characterized.

Materials and Methods

Isolation and identification of lactic acid bacteria

To isolate lactic acid bacteria with antimicrobial activity against MRSA, 10 g of fermented shrimp were added to 10 ml of 1.0% (w/v) peptone-added, sterilized distilled water, suspended well, and then cultured in de Man, Rogosa and Sharpe (MRS) (Difco, USA) agar plates containing 5% (w/v) NaCl, 0.1% (w/v) nystatin (Merck, USA) and 0.1% (w/v) polymyxin B (Duchefa, Netherlands) at 30°C for one day. Genomic DNAs were purified from isolated and cultured cells using a Wizard Genomic DNA Isolation Kit (Promega, USA). The 16S rRNA gene

of isolates was amplified using 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGH TAC CTT GTT ACG ACT T-3'), and sequenced for identification with the GenBank database using the BLAST program (<http://www.ncbi.nlm.nih.gov/>).

Growth characterization of lactic acid bacteria

In order to determine the optimal pH level and NaCl concentration for the growth of the isolated lactic acid bacteria, S1, S2 and S3 strains were cultured in 100 ml of an MRS broth medium (Difco) at various pH levels or at various NaCl concentrations. The pH levels of the media were adjusted with 1 N HCl or 1 N NaOH. After overnight culture, optical densities of strains S1, S2 and S3 were measured using a spectrophotometer (Model: X-ma 3000, Human Corporation, Korea) at 600 nm.

Antibacterial activities of the isolated lactic acid bacteria

The antibacterial activities of strains S1, S2 and S3 against MRSA, *S. aureus*, *Bacillus cereus* and *Escherichia coli* were confirmed by the paper disc method. All four strains were grown at 37°C in 4 ml of tryptic soy (TS) broth media (Difco). After overnight culture, each strain was smeared on TS agar plates, and paper discs (6 mm) were placed on the plates, and then 10 µl of overnight-cultured broths of strains S1, S2 and S3 in 4 ml of an MRS broth media containing 5% (w/v) NaCl were added onto the discs. Ampicillin (10 mg/ml) and kanamycin (30 mg/ml) were used for positive controls and an MRS broth medium containing 5% (w/v) NaCl was used for a negative control.

Preparation and characterization of the bacteriocin

In order to prepare the bacteriocin sample produced by *Weissella* sp. S3 that showed the strongest antibacterial activity against MRSA, strain S3 was cultured in 200 ml of an MRS broth medium at 30°C and 250 rpm for 24 h. Centrifugation of the culture fluid at 3,000 ×g and 4°C for 15 min was performed to recover the supernatant. Six hundred ml of acetone was added to 200 ml of culture supernatant, cooled to 4°C, and then left at -20°C for 24 h. Precipitated proteins were collected by centrifugation at 3,000 ×g and 4°C for 15 min, and then lyophilized. The lyophilized sample was dissolved in an MRS broth medium and used.

To investigate the stabilities of the bacteriocin sample

produced by *Weissella* sp. S3 against heat treatments, the antibacterial activities of the solutions heat-treated for 30 min at 20°, 40°, 60°, 80° and 100°C were measured by the disc diffusion method against MRSA and compared with no treatment.

To figure out the stabilities of the bacteriocin sample produced by *Weissella* sp. S3 against pH changes, the bacteriocin solutions were mixed with the same amounts of buffer solutions at pH 3–5 (20 mM sodium acetate), pH 7 (20 mM Tris-HCl), and pH 9–11 (20 mM GTA). After being placed at room temperature for 24 h, antibacterial activities were measured by the disc diffusion method against MRSA and compared with no treatment.

To determine the stabilities of the bacteriocin sample produced by *Weissella* sp. S3 against organic solvent treatments, the bacteriocin solutions were mixed with the same amounts of organic solvents, such as acetone, chloroform, hexane, ethanol, isopropanol, and methanol. After being placed at 30°C for one hour, antibacterial activities were measured by the disc diffusion method against MRSA and compared with no treatment.

Tricine SDS-PAGE was performed according to Schagger's method [14] to estimate the molecular weight of the bacteriocin produced by *Weissella* sp. S3. The bacteriocin solution was mixed with the same volume of a tricine sample buffer and then heated at 80°C for three minutes. Electrophoresis was performed in 10% (w/v) polyacrylamide gel. After the electrophoresis was completed, the gel was stained for one hour using a Gel-Code Blue Stain Reagent (Thermo Scientific, USA). A

Dokdo-Mark Protein Size Marker (ELPIS Biotech, Korea) was used for the protein size marker.

Results and Discussion

Isolation and identification of lactic acid bacteria

Initially, many salted or Korean traditional fermented foods were used to screen for the antibacterial activity of lactic acid bacteria; however, only fermented shrimp harbored those bacteria in this study. Many bacterial colonies were obtained from the fermented shrimp on MRS agar plates containing 5% (w/v) NaCl, 0.1% (w/v) nystatin and 0.1% (w/v) polymyxin B. Nystatin is an antifungal agent and polymyxin B is an antibacterial agent against Gram-negative bacteria. So, we could selectively cultivate Gram-positive bacteria, including lactic acid bacteria, from the fermented shrimp using a selective medium. Since the culture plates contained 5% (w/v) NaCl, we can also selectively cultivate halophilic or halotolerant bacteria from the fermented shrimp. Finally, three strains were selected after measuring their antibacterial activity against MRSA. The 16S rRNA gene of strains S1, S2, and S3 were amplified by PCR and about 1.5 kb fragments were sequenced.

BLAST was used to search for the sequencing results of 16S rRNA gene of strains S1, S2 and S3 and all three strains were found to be most similar to *Weissella cibaria* with 97% similarity or higher. Therefore, the isolated strains were named as *Weissella* sp. S1, S2, and S3. Phylogenetic trees are shown in Fig. 1. *Weissella cibaria* CMU strain has been commercially used as oral

Fig. 1. Neighbor-Joining phylogenetic tree based on almost complete 16S rRNA gene sequences comparing isolated lactic acid bacteria S1, S2 and S3 with other bacteria. The numbers at the branch nodes are percentages of bootstrap values (n = 1,000) and numbers in parenthesis are accession numbers in GenBank.

care probiotics [15], hence the isolated *Weissella* strains could have the potential to become a probiotic.

Growth characterization of lactic acid bacteria

Each of *Weissella* sp. S1, S2, and S3 grew well between pH 5 and pH 9 (Fig. 2). Rather low growth was shown at pH 4, 10, and 11. *Weissella* sp. S1, S2 and S3 showed optimal growths at pH 7, 9 and 8, respectively. None of the three bacteria was able to grow at pH 3. At pH 10 and 11, *Weissella* sp. S3 showed lower growth rates than those of *Weissella* sp. S1 and S2. These results were the same as the results of the previous study of lactic acid bacteria from kimchi showing highest growth at pH 7 and no growth at pH 3 [12].

All three *Weissella* sp. grew well at 5% (w/v) NaCl (Fig. 3). Moderate growth inhibitions were shown in all three *Weissella* sp. at 7% (w/v) NaCl and strong growth inhibitions were shown at 9% (w/v) NaCl. No growth was exhibited at 11% (w/v) NaCl. These data suggest that

Fig. 2. Effects of pHs on growths of *Weissella* sp. S1 (□), S2 (○) and S3 (●).

Fig. 3. Effects of NaCl concentrations on growths of *Weissella* sp. S1 (□), S2 (○) and S3 (●).

Weissella sp. S1, S2 and S3 can be regarded as halotolerant bacteria [12] as bacteriocins producing *Pediococcus pentosaceus* [7].

Antibacterial activities of *Weissella* sp. strains against several bacterial strains

It is known that lactic acid bacteria inhibit the growth of pathogenic bacteria by producing lactic acid, bacteriocin, and hydrogen peroxide [12, 13, 16–18]. The antibacterial activities of *Weissella* sp. S1, S2, and S3 against pathogenic bacteria are shown in Fig. 4. *Weissella* sp. S1, S2, and S3 showed 4.0, 3.5, and 6.5 mm of antibacterial activities against MRSA, respectively. *Weissella* sp. S1, S2, and S3 showed 2.0, 3.0, and 3.5 mm of antibacterial activities against *S. aureus*, respectively (Fig. 4B). *Weissella* sp. S1, S2, and S3 showed 2.0, 1.0 and 2.0 mm of antibacterial activities against *B. cereus*, respectively (Fig. 4C). *Weissella* sp. S1, S2, and S3 showed 0.5, 0.5, and 0.5 mm of antibacterial activities against *E. coli*, respectively (Fig. 4D). Namely each of the three *Weissella* strains showed antibacterial activity to Gram-positive and Gram-negative bacteria, and more actively inhibited the Gram-positive bacteria. Ampicillin (10 mg/ml) and kanamycin (30 mg/ml) each exhibited 4.0 mm of clear

Fig. 4. Antibacterial activities of *Weissella* sp. S1, S2 and S3 against pathogenic bacteria. (A) MRSA, (B) *S. aureus*, (C) *B. cereus*, (D) *E. coli*. (Amp: ampicillin 10 mg/ml, Kan: kanamycin 30 mg/ml, Med: an MRS broth medium containing 5% (w/v) NaCl).

zones against *S. aureus* (Fig. 4B), but showed no clear zones against MRSA (Fig. 4A). Nonetheless, each of *Weissella* sp. S1, S2, and S3 showed antibacterial activities against MRSA, and *Weissella* sp. S3 exhibited the strongest antimicrobial activity (6.5 mm of the clear zone) (Fig. 4A). Therefore, in this study, the bacteriocin produced by *Weissella* sp. S3 was prepared for and used in further experiments.

Preparation and characterization of the bacteriocin

The antimicrobial activities of the bacteriocin produced by *Weissella* sp. S3 after heat treatment against MRSA are shown in Table 1. No dramatic decrease in antimicrobial activity was shown after heat treatments of the bacteriocin at all temperatures tested (Table 1). A heat-treated sample at 100°C exhibited the lowest antimicrobial activity, but still showed antimicrobial activity (49%) (Table 1). Many antibiotics are sensitive to heat, but the bacteriocin produced by *Weissella* sp. S3 is stable to heat. In general, it is known that bacteriocins have a low molecular weight and thus show high stability against heat treatment [19]. It has been reported that the bacteriocin produced by *Lactococcus lactis* YH-10 exhibits remaining antibacterial activity of about 50% when heat treated at 100°C for 60 min [20].

The antimicrobial activities against MRSA of the bacteriocin produced by *Weissella* sp. S3 after exposure at various pH levels are shown in Table 2. No dramatic decrease of antimicrobial activity was shown after changes to the pH level of the bacteriocin from pH 3 to 10 (Table 2). This result is similar to that of the bacteriocin Weissellin A produced by *Weissella paramesenteroides* DX [21]. However, no antimicrobial activity was observed in the sample at pH 11. It has been reported that nucleophiles such as hydroxide ions, deprotonated amines and deprotonated hydroxyl groups react with

Table 2. Effects of the bacteriocin produced by *Weissella* sp. S3 against pH changes on antimicrobial activities against MRSA.

pH	Remaining activity (%)
3	100
5	100
7	75
9	75
10	49
11	0

dehydro residues at high pH to form cross-linkages between molecules or cause chemical transformation [7]. These data indicate that the bacteriocin produced by *Weissella* sp. S3 is stable at a relatively wide range of pH levels.

The antimicrobial activities against MRSA of the bacteriocin produced by *Weissella* sp. S3 after organic solvent treatments are shown in Table 3. The antibacterial activity of bacteriocin was not diminished after treatment with ethanol or isopropanol, while hexane treatment dropped by 51% (Table 3). The bacteriocin produced by *Lactococcus* sp. KD 28 [22], *Pediococcus pentosaceus* KC-007 [7] does not show any decrease in activity in organic solvents, but the bacteriocin produced by *Weissella* sp. S3 decreases slightly in hexane, so it is necessary to be careful with hexane when future research and the development of therapeutic agents is conducted. However, it has been confirmed that ethanol and isopropanol do not affect bacteriocin stability.

Tricine SDS-PAGE electrophoresis was performed to determine the molecular weight of the bacteriocin produced by *Weissella* sp. S3. The bacteriocin exhibited a single band with a molecular weight of about 6 kDa (Fig. 5). The molecular weights of bacteriocins produced by

Table 1. Effects of the bacteriocin produced by *Weissella* sp. S3 against heat treatments on antimicrobial activities against with MRSA.

Heat treatment (°C)	Remaining activity (%)
4	100
37	100
50	75
70	75
100	49

Table 3. Effects of the bacteriocin produced by *Weissella* sp. S3 on the against organic solvent treatments on antimicrobial activities against MRSA.

Organic solvent	Remaining activity (%)
Acetone	75
Chloroform	75
Ethanol	100
Hexane	49
Isopropanol	100
Methanol	75

Fig. 5. Tricine-SDS-PAGE analysis of the bacteriocin sample produced by *Weissella* sp. S3. (size marker; lane M, the bacteriocin sample; lane B)

Weissella sp. varied, with the molecular weight of a bacteriocin of *Weissella* sp. being 6 kDa for *W. cibaria* KMITL-QU21 [23], 62 kDa for *W. hellenica* D1501 [24], 2.7 kDa for *W. confusa* A3 [25], and 4.5 kDa for *W. paramesenteroides* DX [21]. The *Weissella* sp. S3 strain showed the most similar rRNA sequence to that of *W. cibaria* and the molecular weight of the bacteriocin of *Weissella* sp. S3 was 6 kDa, the same as that of *W. cibaria* KMITL-QU21 [23]. However, the bacteriocin of *Weissella* sp. S3 showed antibacterial activity against *E. coli* while *W. cibaria* KMITL-QU21 did not. Hence, *Weissella* sp. S3 would be a novel strain and its antibacterial activity could be used as a biopreservative etc.

We isolated three lactic acid bacteria, which possess antibacterial activity against MRSA (Methicillin Resistant *S. aureus*), from Korean traditional fermented shrimp. The isolated strains were identified as *Weissella* sp. S1, S2 and S3 by 16S rRNA gene sequencing. All strains showed antibacterial activities against food-poisoning bacteria (*Bacillus cereus*, *S. aureus*), *E. coli* and MRSA. Their optimal pH for growth was pH 7.0, 9.0, and 8.0 for *Weissella* sp. S1, S2 and S3, respectively (Fig. 2). Their growth was similar at 0–5% (w/v) NaCl and inversely proportional to 7–11% (w/v) NaCl (Fig. 3). *Weissella* sp. S3 showed the highest antibacterial activities. The bacteriocin produced by *Weissella* sp. S3 was

prepared and characterized. Remaining antibacterial activities were measured after heat treatment and exposure to various pHs and organic solvents. Remaining antibacterial activities were 100% at 4°C and 37°C, 75% at 50°C and 70°C, and 49% at 100°C (Table 1). Remaining antibacterial activities were 100% at pH 3 and 5, 75% at pH 7 and 9, and 49% pH 10 (Table 2). Ethanol and isopropanol did not decrease the antibacterial activity of bacteriocin while acetone, chloroform and methanol decreased 25% of antibacterial activity (Table 3). The molecular weight of the bacteriocin was about 6 kDa by tricine-SDS-PAGE (Fig. 5). These results suggest that *Weissella* sp. S3 could be a probiotic and its bacteriocin could be useful in the development of safe biopreservatives for food or the treatment of MRSA.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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