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ARTICLE

Antilisterial Bacteriocin from *Lactobacillus rhamnosus* CJNU 0519 Presenting a Narrow Antimicrobial Spectrum

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Abstract

A lactic acid bacterium presenting antimicrobial activity against a *Lactobacillus acidophilus* strain used for eradication of acid inhibition was isolated from a natural cheese. The 16S rRNA gene sequence of the isolate best matched with a strain of *L. rhamnosus* and was designated *L. rhamnosus* CJNU 0519. The antimicrobial activity of the partially purified bacteriocin of CJNU 0519 was abolished when treated with a protease, indicating the protein nature of the bacteriocin. The partially purified bacteriocin (rhamnocin 519) displayed a narrow antimicrobial activity against *L. acidophilus*, *Listeria monocytogenes*, and *Staphylococcus aureus* among several tested bacterial and yeast strains. Rhamnocin 519 in particular showed strong bactericidal action against *L. monocytogenes.*

Key words: bacteriocin, *Lactobacillus rhamnosus*, narrow-range spectrum, *Listeria monocytogenes*, probiotics

Introduction

Bacteriocins are proteinaceous antimicrobial substances that inhibit the growth of closely related bacterial species (Drider *et al.*, 2006; Klaenhammer, 1993). Bacteriocins produced from lactic acid bacteria (LAB) have been especially studied because of their safety. Many LAB bacteriocins have been identified and explored as food biopreservatives or antibiotic alternatives (Allen *et al.*, 2014; Bali *et al.*, 2014; Cui *et al.*, 2012; Lohans and Vederas, 2012).

LAB bacteriocins are generally categorized into three classes (Nes and Holo, 2000). Class I (lantibiotics) contains post-translationally modified peptides having multiple thioether crosslinks formed by the addition of the thiols of cysteine to dehydroamino acids like dehydroalanine (from serine) and dehydrobutyrine (from threonine) (Yu *et al.*, 2013). Nisin A and Z from *Lactococcus lactis* ssp. *lactis* is a representative Class I bacteriocin (Ward *et al.*, 1994). Class II contains non-modified and heat-stable bacteriocins which are further divided into three subclasses: strong anti-listerial pediocin-like bacteriocins (subclass IIa), two-peptide bacteriocins (subclass IIb), and other bacteriocins (subclass IIc) (Nes and Holo, 2000). Particularly, the pediocin family share consensus amino acid sequences between 40% to over 70% where YGNGV in the N-terminal sequence is highly conserved between the bacteriocins (Nes and Holo, 2000). Class III contains large heat-labile bacteriocins including helveticin J and enterolysin A (Joerger and Klaenhammer, 1990; Nilsen *et al.*, 2003).

Among the identified bacteriocins, only nisin has been marketed (Lauková *et al.*, 2014). Nisaplin is approved for use in over 50 countries (Lalpuria *et al.*, 2013). Several bacteriocins including pediocin PA-1 are promising candidate for biopreservatives or antibiotic alternatives (Bali *et al.*, 2014; Cotter *et al.*, 2013; Mehta *et al.*, 2013). Nevertheless, these bacteriocins mostly have a broad antimicrobial spectrum against gram-positive bacteria (Rodríguez *et al*., 2002; Ross *et al.*, 1999). While this attribute helps to control a broad range of gram-positive pathogenic bacteria (Mehta *et al.*, 2013), the bacteriocins can also inhibit beneficial gram-positive bacteria, such as LAB (Han *et al.*, 2007; Kwon *et al.*, 2002; Lee *et al*., 2002). Isolation of LAB bacteriocins capable of very targeting inhibition of, for example, food pathogens or spoilage bacteria, is desirable. Among *Lactobacillus rhamnosus* strains, *L. rhamnosus* strain 68 was confirmed to produce a bacteriocin rhamnosin A which was a small, heat-stable, and nonlanthionine-containing peptide and therefore categorized as a class II bacteriocin (Dimitrijević *et al.*, 2009).

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Listeria monocytogenes and *Staphylococcus aureus* are representative food pathogenic bacteria (Gahan and Hill, 2014; Larkin *et al.*, 2009). Particularly, *L. monocytogenes* is a psychrotrophic bacterium that grows at low temperature. Listeriosis outbreaks have been normally linked to consumption of milk products (Markkula *et al.*, 2012; Rossi *et al.*, 2008). *L. monocytogenes* is a threat to dairy food systems and effective control methods are needed. In this study, we isolated a LAB that produces a bacteriocin with a narrow antimicrobial spectrum that includes *L. monocytogenes* and *S. aureus*. Here we report the preliminary results.

Materials and Methods

Bacterial strains and culture conditions

Indicator strains were *L. reuteri* KCTC 3679, *L. casei* CJNU 0588, *L. acidophilus*, *Leuconostoc mesenteroides* CJNU 0147, *Pediococcus acidilactici* K10, *Enterococcus faecium* MK3, *Bacillus licheniformis* 1-B-12, *L. monocytogenes* KCTC 3569, *S. aureus* ATCC 14458, *Escherichia coli* DH5α, and *Saccharomyces cerevisiae* ATCC 24858. ATCC and KCTC strains were purchased from the American Type Culture Collection and Korean Collection for Type Cultures, respectively. LAB, *L. monocytogenes,* and *S. aureus* were cultured at 37°C with no agitation in MRS broth (De Man, Rogosa, and Sharpe) purchased from Difco (Sparks, MD, USA). *B. subtilis* and *E. coli* was cultured in nutrient broth (Difco) and Luria-Bertani (LB) broth (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract, pH 7.0), respectively, at 37°C with shaking. *S. cerevisiae* was cultured in YPD broth (yeast extract 10 g/L, peptone 20 g/L, and dextrose 20 g/L) at 30°C.

Bacteriocin production assay

Bacteriocin production by isolate CJNU 0519 was confirmed by a deferred antagonism assay for viable cells and spot-on-lawn assay for pH-neutralized culture supernatant (Daeschel, 1992; Moon *et al.*, 2000). Briefly, in the deferred antagonism assay, 1 μL of the isolate culture was loaded on MRS agar and incubated for 12 h at 37°C. Top agar (0.7%, w/v) seeded with *L. acidophilus* used for eradication of acid inhibition was overlaid on the agar and incubated for further 12 h, and the inhibitory zone was examined. In the spot-on-lawn assay, the pH of the isolate culture was neutralized with 1 M NaOH and centrifuged at 5,000 *g* for 10 min. The culture supernatant was filtered through 0.2 μm syringe filters (Millipore, USA). The filtrate was concentrated with an acetone extraction method (Chung *et al.*, 2011) and loaded onto a plate that had been overlaid with top agar seeded with *L. acidophilus* and incubated for 12 h at 37°C. The concentrate was also treated with protease (Tokyo chemical, Japan), lipase (Tokyo chemical), and α-amylase (Sigma-Aldrich, USA) for confirmation of proteinaceous antimicrobial peptide (bacteriocin).

Isolate identification

The 16S rRNA gene sequence of CJNU 0519 was analyzed for bacterial identification. The strain was overnight cultured in MRS broth and sent to Macrogen (Korea) for the 16S rRNA gene sequence analysis. The gene sequence was aligned and homology search was performed by a BLAST program (http://www.ncbi.nlm.nih.gov/).

Preparation of partially purified bacteriocin

Partially purified bacteriocin was prepared using the acetone extraction method (Chung *et al.*, 2011). As above mentioned, an overnight culture of CJNU 0519 was neutralized with 1 M NaOH, centrifuged at 5000 *g* for 10 min, and filtered. The culture supernatant was mixed with acetone at a ratio of 1:3 and stored at -20°C for 3 h. The mixture was centrifuged at 5000 *g* for 15 min. The supernatant was recovered and evaporated with a rotary evaporator. The partially purified bacteriocin was stored at -20°C until use. The bacteriocin activity expressed as arbitrary units (AU)/mL was defined as the reciprocal of the highest two-fold dilution showing an inhibition zone (Daeschel, 1992).

Antimicrobial spectrum

The antimicrobial spectrum of *L. rhamnosus* CJNU 0519 cells and the partially purified bacteriocin (rhamnocin 519) was investigated using the aforementioned bacteriocin assays. Indicators were the gram-positive bacteria *L. reuteri* KCTC 3679, *L. casei* CJNU 0588, *L. acidophilus*, *L. mesenteroides* CJNU 0147, *P. acidilactici* K10, *E. faecium* MK3, *B. licheniformis* 1-B-12, *L. monocytogenes* KCTC 3569, and *S. aureus* ATCC 14458; gram-negative bacterium *E. coli* DH5α; and yeast *S. cerevisiae* ATCC 24858.

Action mode of rhamnocin 519

An overnight culture of *L. monocytogenes* KCTC 3569 was inoculated in 5 mL of peptone water (0.1%, w/v) and cultured at 37°C for 21 h. At 3 h, 150 and 300 AU/mL of partially purified rhamnocin 519 were added into the inoculum, respectively. The viable cell count of *L. monocyt-*

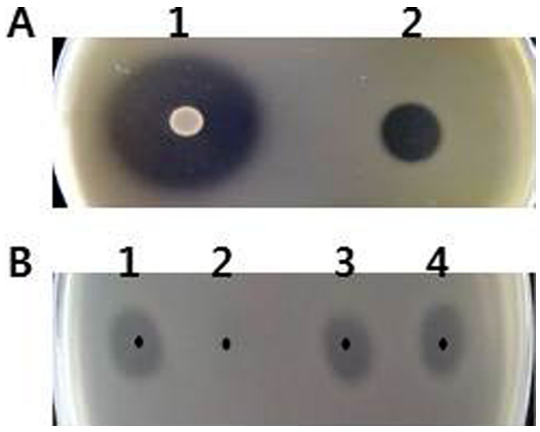

Fig. 1. Bacteriocin activity of lactic acid bacterium strain CJNU 0519. A. Deferred antagonism assay (1) for viable cells and spot-on-lawn assay (2) for concentrated partially purified bacteriocin. B. Spot-on-lawn assay for concentrated partially purified bacteriocin (1) and the bacteriocin treated with protease (2), lipase (3), and α-amylase. *Lactobacillus acidophilus* was used as the indicator.

ogenes was analyzed on MRS agar plates at intervals.

Results and Discussion

Bacteriocin production from CJNU 0519

LAB CJNU 0519 isolated from a natural cheese was confirmed to produce a bacteriocin using the deferred antagonism assay for viable cells and spot-on-lawn assay for the partially purified bacteriocin. As shown in Fig. 1, CJNU 0519 viable cells presented inhibition zone against *L. acidophilus* used for eradication of acid inhibition, and the partially purified bacteriocin also showed antimicrobial activity against the indicator strain. The bacteriocin was treated with protease, lipase, and α-amylase. Protease abolished the antimicrobial activity of the bacteriocin, indicating its proteinaceous nature. LAB can produce antimicrobial substances including organic acids and hydrogen peroxide, as well as bacteriocins (Pawlowska *et al.*, 2012). Therefore, it is a prerequisite to check whether the antimicrobial activity of an isolate is caused by a bacteriocin.

Identification of CJNU 0519

The 16S rRNA gene sequence of CJNU 0519 was analyzed for bacterial identification. The gene sequence was aligned and homology search was performed. The sequence best matched (99% identity) that of *L. rhamnosus* ATCC 8530 (GenBank accession no., CP003 094.1). The isolate was designated *L. rhamnosus* CJNU 0519. The bacteriocin produced from *L. rhamnosus* CJNU 0519 was designated rhamnocin 519. A phylogenetic tree based on 16S rRNA gene sequences of CJNU 0519 and related type strains was shown in Fig. 2.

Antimicrobial spectra of *L. rhamnosus* CJNU 0519 and rhamnocin 519

The antimicrobial spectra of *L. rhamnosus* CJNU 0519

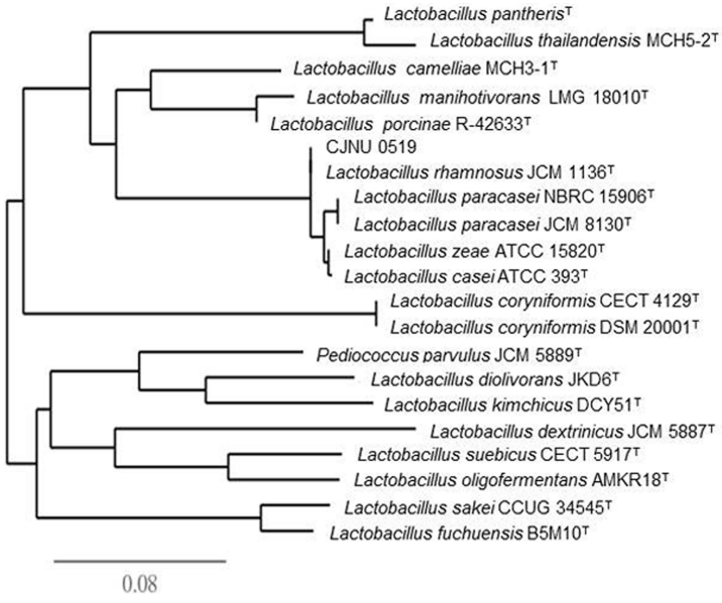

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequences of CJNU 0519 and related type strains. The branch length is proportional to the number of substitutions per site and the tree was constructed based on an algorithm from http://phylogeny.lirmm.fr.

Table 1. Antimicrobial spectrum of *L. rhamnosus* CJNU 0519 cells and partially purified rhamnocin 519

Target strain			Deferred antagonism assay[1)]	Spot-on-lawn assay[2)]
Gram-positive	Lactic acid bacteria	*Lactobacillus reuteri* KCTC 3679	-	-
		Lactobacillus casei CJNU 0588	-	-
		Lactobacillus acidophilus	+	+
		Leuconostoc mesenteroides CJNU 0147	-	-
		Pediococcus acidilactici K10	+	-
		Enterococcus faecium MK3	-	-
	Bacillus	*B. licheniformis* 1-B-12	+	-
	Pathogenic bacteria	*Listeria monocytogenes* KCTC 3569	+	+
		Staphylococcus aureus ATCC 14458	+	+
Gram-negative		*Escherichia coli* DH5α	-	-
Yeast		*Saccharomyces cerevisiae* ATCC 24858	-	-

[1)]Deferred antagonism assay for *L. rhamnosus* CJNU 0519 cells.
[2)]Spot on the lawn assay for partially purified rhamnocin 519.

cells and partially purified rhamnocin 519 was investigated against several gram-positive bacteria, gram-negative bacteria, and yeast. *L. rhamnosus* CJNU 0519 cells inhibited the growth of *L. acidophilus*, *P. acidilactici* K10, *B. licheniformis* 1-B-12, *L. monocytogenes* KCTC 3569, and *S. aureus* ATCC 14458, while the partially purified rhamnocin 519 inhibited *L. acidophilus*, *L. monocytogenes* KCTC 3569, and *S. aureus* ATCC 14458 (Table 1). The inhibitory effect of *L. rhamnosus* CJNU 0519 cells on the growth of *P. acidilactici* K10 and *B. licheniformis* 1-B-12 might be due to organic acids from the cells. Rhamnocin 519 has a narrow antimicrobial spectrum, which could be exploited to control food-borne pathogenesis caused by *L. monocytogenes* or *S. aureus*. This selective targeting could minimalize the growth inhibition of beneficial bacteria for human health including LAB. Effects of broad- and narrow-range antimicrobials on enteropathogenic *Clostridium difficile* and microbial diversity in a model of the human distal colon have been compared (Rea *et al*., 2011). In the study, broad-range antimicrobials including vancomycin, metronidazole, and the bacteriocin lacticin 3147 significantly reduced the viable numbers of *C. difficile* and other members of the human gut microbiota. The narrow-range antimicrobial bacteriocin thuricin CD produced by *B. thuringiensis* also significantly decreased *C. difficile* viability, but had no significant impact on microbial composition. The study highlighted the value of narrow-range antimicrobial bacteriocins and their potential as biopreservatives or antibiotic alternatives.

Action mode of rhamnocin 519

Action mode of rhamnocin 519 was presented in Fig. 3. Partially purified rhamnocin 519 (150 and 300 AU/mL) was added to 5 mL of peptone water (0.1%, w/v) previously inoculated with an overnight culture of *L. monocytogenes* KCTC 3569. Before the addition of rhamnocin 519, the initial viable counts of *L. monocytogenes* were almost the same between samples. Subsequently, significant decreases were evident at 3 h in the bacteriocin containing samples. The initial cell count of bacteriocin-free sample was 5.75 Log colony forming units (CFU)/mL and reached 5.08 Log CFU/mL at 21 h. Similarly, the initial cell count of sample containing 150 AU/mL rhamnocin 519 was 6.06 Log CFU/mL, with a decrease to 0.33 Log CFU/mL at 3 h and no detectable viable cells at 9 h. The initial cell count of sample containing 300 AU/mL rhamnocin 519 was 5.84 Log CFU/mL, with no viable cells detected at 3 h. These results indicate a potent anti-

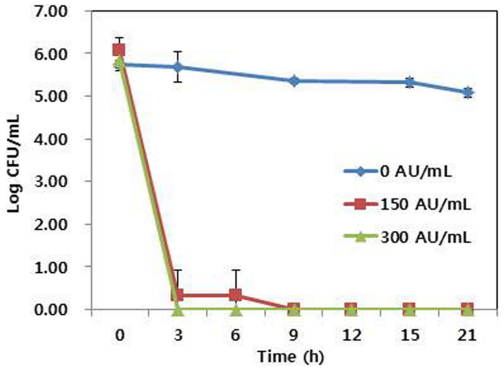

Fig. 3. Action mode of partially purified rhamnocin 519 against *Listeria monocytogenes*. Abbreviations: 0 AU/mL, *L. monocytogenes* was inoculated in peptone water (0.1%, w/v); 150 and 300 AU/mL, 150 and 300 AU/mL of partially purified rhamnocin was added to the inoculum, respectively at 3 h. The values are presented as mean ± SD.

listerial activity of rhamnocin 519; the activity is consistent with categorization as a Class IIa bacteriocin (Nes and Holo, 2000).

LAB bacteriocins are of interest because of their safety and antimicrobial potential against food-borne pathogens and spoilage bacteria. Many LAB bacteriocins have been identified and explored as food biopreservatives or antibiotic alternatives (Allen *et al.*, 2014; Bali *et al.*, 2014; Cui *et al.*, 2012; Lohans and Vederas, 2012). Most bacteriocins have a broad-range antimicrobial spectrum, which can deleteriously influence the human gut microbiota and inhibit bacteria that are beneficial for human health (Rea *et al.*, 2011). Bacteriocins with a narrow antimicrobial spectrum against specific food-borne pathogens or spoilage bacteria could be more promising therapeutic agents or natural preservatives.

In this study, the novel LAB, *L. rhamnosus* CJNU 0519, was shown to produce the bacteriocin rhamnocin 519, which displayed a narrow antimicrobial spectrum that included *L. monocytogenes* and *S. aureus*. Particularly, rhamnocin 519 strongly inhibited the growth of *L. monocytogenes* highlighting the potential of *L. rhamnosus* CJNU 0519 in the control of food-borne pathogens. Additionally, rhamnocin 519 could be a promising candidate biopreservative or antibiotic alternative. Further analyses, such as optimal culture condition for bacteriocin mass production, biochemical properties of rhamnocin 519, and identification of corresponding genes for the bacteriocin production, will be performed in the near future.

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