Short communication: In vivo screening platform for bacteriocins using *Caenorhabditis elegans* to control mastitis-causing pathogens

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ABSTRACT

This study aimed to develop an in vivo screening platform using *Caenorhabditis elegans* to identify a novel bacteriocin for controlling the mastitis-causing pathogen *Staphylococcus aureus* strain RF122 in dairy cows. Using *Bacillus* spp. isolated from traditional Korean foods, we developed a direct in vivo screening platform that uses 96-well plates and fluorescence image analysis. We identified a novel bacteriocin produced by *Bacillus licheniformis* strain 146 (lichenicin 146) with a high in vivo antimicrobial activity using our liquid *C. elegans*–*Staph. aureus* assay. We also determined the characteristics of lichenicin 146 using liquid chromatography-mass spectrometry and confirmed that it shared homologous sequences with bacteriocin family proteins. In addition, RNA-sequencing analysis revealed genes encoding cell surface or membrane proteins (*SAB0993c*, *SAB0150*, *SAB0994c*, and *SAB2375c*) that are involved in the bactericidal activity of lichenicin 146 against *Staph. aureus* strain RF122 infection as well as those encoding transcriptional regulators (*SAB0844c* and *SAB0133*). Thus, our direct in vivo screening platform facilitates simple, convenient, cost-effective, and reliable screening of potential antimicrobial compounds with applications in the dairy field.

Key words: bacteriocin, *Caenorhabditis elegans*, in vivo screening platform, mastitis-causing pathogen, *Staphylococcus aureus*

Short Communication

Mastitis in dairy cows is defined as an inflammation of the udder caused by a variety of microorganisms

that gain access to the interior of the mammary glands through the teat canal. It is a chronic wasting disease and leads to substantial economic losses in dairy farms due to reproductive disorders, reduced milk production, and a decreased lactation period in dairy cows. Moreover, it poses a serious threat to the health of consumers because it can result in degradation of the quality of raw milk (Schukken et al., 2011).

Diverse microorganisms, such as bacteria, mycoplasma, yeasts, and algae, have been implicated as causes of mastitis (Watts, 1988). In general, 4 common bacteria (i.e., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*) cause 90 to 95% of all infections in dairy industry (Miles et al., 1992); among them, *Staph. aureus* is the main mastitis-causing pathogen (Ikawaty et al., 2009). To treat and prevent mastitis infections, the use (and misuse) of antibiotics has been widespread in clinical and veterinary medicine during recent decades (Sears et al., 1995). The number of somatic cells and the use of antibiotics are closely related to mastitis (Ruegg and Tabone, 2000; Mungube et al., 2005); however, the use of antibiotics to control mastitis should be carefully considered because it may lead to antibiotic residues in the bodies of treated cows or transfer of antibiotics to raw milk through mammary glands and blood. Thus, residual antibiotics may accumulate in the meat and milk from treated cows. The presence of residual antibiotics in milk is not only undesirable from a public health perspective, but can also cause problems in fermented dairy products such as yogurt, cheese, and ice cream (Allison, 1985). Therefore, many methods have been developed to identify new natural antibacterial substances, including bacteriocins, which are generally defined as antimicrobial peptides that are synthesized ribosomally by bacteria and secreted into the extracellular milieu, where they usually act against closely related species without affecting the producer strain (Klaenhammer, 1993); thus, bacteriocins may be useful alternatives to antibiotics for the control of mastitis. These natural bacteriocins are particularly suitable

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given their use and acceptance as preservatives in foods (O'Shea et al., 2013).

Various methods have been employed to screen bacteriocins from probiotic bacteria. Of these, the most frequent method is simple in vitro assays, including well diffusion, because of the difficulty involved in screening active bacteriocins from several candidates in direct animal models. In addition, because of concerns over animal welfare, it is difficult to perform in vivo toxicity and survival assays using animals (Dawkins, 2012). To overcome these limitations, we developed a direct in vivo screening platform using *Caenorhabditis elegans* as a new in vivo model. *Caenorhabditis elegans* is a small, living soil nematode that feeds on bacteria and has been extensively used as an experimental system in biological studies because of its simplicity, transparency, ease of cultivation, and suitability for genetic analysis (Riddle et al., 1997). *Caenorhabditis elegans*–pathogen infection models have been used in several studies exploring pathogenesis as well as in screens for compounds with antimicrobial activity (Irazoqui et al., 2010; Rajamuthiah et al., 2014). The aim of this study was to develop a direct in vivo screening platform using *C. elegans* for bacteriocins produced by *Bacillus* spp. from traditional Korean foods to control mastitis-causing pathogens and to characterize the novel bacteriocin identified by proteomic and transcriptomic analysis.

Staphylococcus aureus strain RF122 provided by Sergine Even (INRA, Rennes, France; Bouchard et al., 2013) was used as the representative mastitis-causing strain in this study. *Bacillus* spp. were isolated from traditional Korean foods such as soy sauces, red pepper paste, and soybean paste (Yun et al., 2014). *Staphylococcus aureus* was cultured at 37°C in brain heart infusion (BHI) medium (BD Biosciences, Sparks, MD) for 18 h at 180 rpm. *Bacillus* spp. were cultured at 30°C in Luria Bertani or de Man, Rogosa, and Sharpe (MRS) medium (BD Biosciences). Cultures were maintained at –80°C in cryoprotectant containing 50% glycerol for long-term storage. All strains were subcultured 2 times before the experimental analysis. The *C. elegans* strain used in this study was *glp-4(bn2);sek-1(km4)* double mutant strain (Kim and Mylonakis, 2012). This nematode strain was routinely maintained on nematode growth medium plates seeded with *Escherichia coli* OP50, as described previously (Park et al., 2013).

First, to prepare bacteriocins from *Bacillus* spp., bacterial strains were cultured in MRS broth at 30°C and 150 rpm and then harvested by centrifugation at $3,000 \times g$ for 10 min (4°C). Cell-free supernatant was collected and filtered through a sterile 0.2- μ m syringe filter (Sartorius, Gottingen, Germany). The antimicrobial activity of *Bacillus* spp. was then determined with

antibiotic-resistant pathogens, including methicillin-resistance *Staph. aureus* (MRSA) strain MW2 and COL and vancomycin-resistant *Enterococcus faecalis* (VRE) strain VS583 (Kim and Mylonakis, 2012), as well as mastitis-causing *Staph. aureus* strain RF122 as an indicator strains using the disk diffusion method, as described previously (Yun et al., 2014). Antimicrobial activity was determined based on the production of a clear zone of inhibition on the agar.

Next, to develop the in vivo screening platform for bacteriocins using *C. elegans*, the *C. elegans glp-4(bn2);sek-1(km4)* worms at the young adult or L4-stage were infected with *Staph. aureus* RF122, which had been cultured overnight in BHI medium at 37°C. Synchronized young adult or L4 nematodes were transferred to plates seeded with *Staph. aureus* strain RF122 for 24 h at 25°C. After washing 3 times with M9 buffer (3.0 g of KH_2PO_4 , 6.0 g of Na_2HPO_4 , 0.5 g of NaCl, and 1.0 g NH_4Cl for 1 L), the nematodes were transferred into the wells of 96-well plates using a washer or dispenser (EL406, Bio-Tek; approximately 15–20 worms in each well). Each well contained 200 μ L of assay medium (20% bacteriocin and 80% M9 medium, vol/vol). In addition, chloramphenicol at a concentration of 90 μ g/mL was used as a positive control and MRS broth alone was employed as a negative control. After incubating for 3 d in a humidified incubator at 25°C, the pathogen and other remains were washed from the wells using an automated washer. To selectively stain dead worms, SYTOX Orange (Life Technologies, Carlsbad, CA) was dispensed into each well at a final concentration of 1.0 μ M. The plates were then sealed and incubated at 25°C in a humidified incubator. After incubating for 24 h, the plates were visualized using a fluorescent microscope (IX53, Olympus, Tokyo, Japan) to capture both transmitted light and fluorescent images (Figure 1).

The characteristics of the novel bacteriocin were also determined using proteome analysis. After ultrafiltration through 3-, 10-, and 30-kDa membranes (AmiconUltra-15, Millipore Corp., Billerica, MA), a fraction of the target bacteriocin was separated by Tricine-SDS-PAGE (Schägger and Von Jagow, 1987). The gel was fixed and one half of the gel was stained with Coomassie blue R250 (Precision Plus Protein Dual Xtra Standards, Bio-Rad, Hercules, CA), before destaining, and the position of the active bacteriocin was determined. To localize bacteriocin activity, the second half of the gel was washed with deionized water, overlaid with BHI soft agar in a plate inoculated with 1% of the overnight-cultured *Staph. aureus* RF122 as an indicator strain, and then incubated overnight at 37°C. Furthermore, the target bacteriocin was identified using liquid chromatography-tandem mass spectrometry

(LC-MS/MS; model 4800; Applied Biosystems), as previously described (Lee et al., 2014). The MASCOT algorithm (Matrix Science, Boston, MA) was used to identify peptide sequences present based on a protein sequence database.

To explore gene regulation in *Staph. aureus* strain RF122 in the presence or absence of the selected bacteriocin, whole-transcriptome analysis was performed by RNA-sequencing (RNA-Seq). *Staphylococcus aureus* strain RF122 was inoculated into 20 mL of assay medium with or without the selected bacteriocin (20% bacteriocin and 80% M9 medium, vol/vol). The flasks were statically incubated at 25°C for 6 h. The cells were then harvested by centrifugation at $9,000 \times g$ for 5 min (4°C). To extract high-quality bacterial RNA, an RNeasy Protect kit (Qiagen, Hilden, Germany) was used with an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The Ribo-Zero rRNA removal kit (Epicentre, Madison, WI) was used for ribosomal RNA depletion according to manufacturer instructions.

Libraries for Illumina sequencing were made with the TruSeq Stranded mRNA sample prep kit (Illumina, San Diego, CA) following the manufacturer's protocol. The RNA-seq was performed on the Illumina HiSeq 2500 platform using single-end 50-bp sequencing. The genome sequence of *Staph. aureus* strain RF122 was retrieved from the NCBI database (accession number NC_007622.1). The relative transcript abundance was measured as reads per kilobase of exon sequence per million mapped sequence reads (Mortazavi et al., 2008). Visualization of mapping results and differentially expressed gene analysis were performed using CLRNaseq program (Chunlab, Seoul, Korea).

For exploring antibacterial activity of selected bacteriocin to dairy environments, we obtained fresh soil samples from local dairy farms (Jeollabuk-do, Korea). The soil samples (10 g) were suspended with M9 buffer with or without selected bacteriocin and incubated at 25°C for 24 h with agitation. And then, each suspension were plated on the standard plate count (BD Biosci-

Figure 1. Schematic of procedure of in vivo screening platform for bacteriocins using *Caenorhabditis elegans*. RF122 = *Staphylococcus aureus* strain RF122; SYTOX = SYTOX orange stain (Life Technologies, Carlsbad, CA). Color version available online.

ences) agar for enumeration of total bacteria, including potential mastitis-causing pathogens.

As previously reported (Yun et al., 2014), among 300 strains isolated from traditional Korean foods, the cell-free filtered supernatants of selected health-promoting *Bacillus* spp. had specific antibacterial activities against various gram-positive pathogens including *Micrococcus luteus* and *Bacillus cereus*. In the present study, we used the disk diffusion method to show that, among the 300 isolated strains, 19 strains of *Bacillus* spp. possessed inhibitory activities against mastitis-causing *Staph. aureus* strain RF122 (data not shown). In particular, a bacteriocin isolated from *B. licheniformis* strain 146 (isolated from soybean paste *Doenjang*) had an antimicrobial activity against *Staph. aureus* strain RF122. Importantly, we showed that this bacteriocin possessed additional antimicrobial activity against to antibiotics-resistant pathogens, such as MRSA and VRE strains. Antibiotics-resistant microbes have frequently been reported in dairy environments, even though they appeared with low prevalence. Among them, MRSA and VRE strains poses a serious threat to emergence of bovine mastitis in a dairy herd (Haran et al., 2012). To our knowledge, the current study is the first description of a dairy-associated bacteriocin that possess strong antimicrobial activity against mastitis-causing *Staph. aureus* as well as MRSA and VRE strains.

Further, using the in vivo *C. elegans* liquid assay, we determined whether the antimicrobial substance produced by *Bacillus* spp. could enhance the viability

of nematodes in an in vivo environment. We used MRS broth alone and chloramphenicol (90 µg/mL), which is a common antibiotic used in the treatment of bovine mastitis (Fitzgerald et al., 2001), as negative and positive controls, respectively. To distinguish dead worms from live worms, we employed the SYTOX staining method, which is a convenient method for counting dead *C. elegans* under a fluorescent microscope because SYTOX Orange is excluded by living cells whereas it readily enters cells with damaged membranes, thereby staining dead worms (Rajamuthiah et al., 2014). Using the *C. elegans*–*Staph. aureus* liquid assay, the survival rates of 18 out of 19 screened strains were approximately less than 10%, including 18 strains that exhibited inhibitory activity in the in vitro disk diffusion assay. Based on the number of SYTOX-stained nematodes (dead worms) in the fluorescence images, *B. licheniformis* strain 146 specifically enhanced the survival of *C. elegans* when infected with mastitis-causing *Staph. aureus* strain RF122 (survival rate of 74% compared with the chloramphenicol control; Figure 2A). In agreement with the results obtained using the in vivo platform, the antimicrobial activity of *B. licheniformis* strain 146 was confirmed based on its effect on the lifespan of *C. elegans* in a manual killing assay, as previously described (Kim and Mylonakis, 2012; Figure 2B). According to the results of our study, compared with the in vitro disk diffusion assay (Figure 3A), the liquid *C. elegans*–pathogen infection assay facilitates more effective screening to identify potential bacterio-

Figure 2. *Caenorhabditis elegans*-based in vivo platform for identifying bacteriocins produced by *Bacillus licheniformis* strain 146 (lichenicin 146) to combat mastitis-causing *Staphylococcus aureus* strain RF122. (A) Bright-field or SYTOX (Life Technologies, Carlsbad, CA) fluorescent images of *C. elegans* infected with *Staph. aureus* strain RF122 in the presence of lichenicin 146 for 3 d. In the bright-field images, live nematodes have a sinusoidal shape in the liquid infection assay, whereas dead nematodes appear as straight and rigid rods. Only dead worms were stained in the SYTOX-fluorescent images. (B) Lifespan of *C. elegans* infected with *Staph. aureus* strain RF122 in the presence of lichenicin 146 for 20 d using the manual killing method. Survival statistics: $P < 0.0001$ for both chloramphenicol and lichenicin 146 compared with the negative control. As the negative and positive controls, we used medium alone and chloramphenicol at a concentration of 90 µg/mL, respectively. Color version available online.

Figure 3. Detection of the antimicrobial activity of lichenicin 146 produced by *Bacillus licheniformis* strain 146. (A) Inhibitory activity of lichenicin 146 against mastitis-causing *Staphylococcus aureus* strain RF122 and antibiotic resistant pathogens including methicillin-resistant *Staph. aureus* (MRSA) strain MW2 and CLO, and vancomycin-resistant *Enterococcus faecalis* (VRE) strain VS583 according to the disk diffusion method. (B) Tricine-SDS-PAGE analysis of the lichenicin 146. Gel stained with Coomassie blue stain (M, Precision Plus Protein Dual Xtra Standards, Bio-Rad, Hercules, CA); lane 1, concentrated lichenicin 146 using cut-off membrane. In addition, the gel was overlaid with *Staph. aureus* strain RF122 cells in brain heart infusion soft agar and incubated overnight at 37°C (lane O). Asterisk indicates the zone of inhibition with lichenicin 146 against *Staph. aureus* strain RF122. (C) The enumeration of total bacteria (including potential mastitis-causing pathogens) in dairy soil environment with or without lichenicin 146. Data are expressed as means $\pm$ SD of 3 experiments. The asterisk indicates statistical significance determined using a Student's *t*-test ($P < 0.05$). Color version available online.

cins that could combat mastitis-causing *Staph. aureus*. The bacteriocin produced by *B. licheniformis* strain 146 is referred to as lichenicin 146, and it was tested in further experiments.

To determine the characteristics of lichenicin 146, we performed SDS-PAGE and LC-MS/MS analyses. The

Tricine-SDS-PAGE results showed that lichenicin 146 had an estimated molecular mass of approximately 10 kDa and the active fraction possessed an antibacterial activity against *Staph. aureus* strain RF122 in a gel overlay assay (Figure 3B). To characterize lichenicin 146 more precisely, proteome analysis was conducted

Table 1. Partial list of differentially expressed genes in mastitis-causing *Staphylococcus aureus* strain RF122 when treated with lichenicin 146 produced by *Bacillus licheniformis* strain 146 for 6 h

Gene number	Fold change	Description
Transcription		
<i>SAB0844c</i>	240.4	Transcriptional regulator
<i>SAB0133</i>	220.5	Transcriptional regulator
<i>SAB2007c</i>	8.0	Predicted transcriptional regulator
<i>SAB0420c</i>	6.9	Transcriptional regulator
<i>SAB0063</i>	5.9	Predicted transcriptional regulator
<i>SAB2581</i>	5.4	Cold shock protein
Replication, recombination and repair		
<i>SAB1521c</i>	13.9	DNA repair protein
Cell wall/membrane/envelope biogenesis		
<i>SAB0357</i>	39.8	Spore coat protein
<i>SAB1896c</i>	38.1	Spore coat protein
<i>SAB0993c</i>	21.5	Cell surface protein
<i>SAB0866</i>	10.0	Competence protein
<i>SAB0150</i>	6.2	Membrane proteins related to metalloendopeptidase
<i>SAB0887</i>	5.6	Membrane proteins TerC, possibly involved in tellurium resistance
<i>SAB0994c</i>	4.3	Cell surface protein
<i>SAB0100</i>	-4.7	Membrane protein involved in the export of O-antigen and teichoic acid
<i>SAB0906c</i>	-4.3	Predicted membrane protein
<i>SAB1798</i>	-4.3	Predicted membrane protein
<i>SAB1325c</i>	-3.7	Predicted membrane protein
<i>SAB0982</i>	-3.5	Predicted membrane protein
<i>SAB1863c</i>	-3.5	Predicted membrane protein
<i>SAB0290c</i>	-3.2	Predicted membrane protein
<i>SAB1386c</i>	-3.1	Predicted membrane protein
<i>SAB1044</i>	-3.4	Protein required for the initiation of cell division
Defense mechanisms		
<i>SAB1681c</i>	-15.0	Lantibiotic modifying enzyme
Function unknown		
<i>SAB2154c</i>	-3.3	Uncharacterized conserved protein
<i>SAB1421c</i>	-2.7	Uncharacterized conserved protein

with LC-MS/MS, which detected a protein with sequence coverage of 40% (the searched peptide number was 126 and the matched peptide number was 51). Using BLAST similarity search via the NCBI database, the protein sequence of lichenicin 146 was found to be highly homologous with a bacteriocin family protein (KJE33240.1). *Bacillus* spp., including *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus amyloliquefaciens*, and *B. licheniformis*, produce low-molecular-weight bacteriocins (smaller than 10 kDa) that belong to the class II bacteriocin family (Barbosa et al., 2015). Thus, we propose that lichenicin 146 may also belong to the class II bacteriocin family due to its low molecular weight. Further research is needed to determine the specific anti-*Staph. aureus* feature of lichenicin 146 based on structural analysis coupled with crystallography.

Several reports indicated that nisin, as one of well-established bacteriocin, could be applied on the bovine mastitis (Malvisi et al., 2016), but they were critically limited, as nisin are only effective at low pH. The results from bacterial-counting assay showed that total bacteria (including potential mastitis-causing pathogens) existed in dairy soil samples but was significantly

reduced by the presence of lichenicin 146 (Figure 3C); hence, lichenicin 146 possesses antimicrobial activity on the neutral pH soil environment in dairy farms and influences the emergence of potential mastitis-causing pathogens. Importantly, recent studies showed that *Bacillus* spp., including *B. licheniformis*, confer significant potential probiotic agents for feed additives (Sorokulova et al., 2008). Indeed, supplementation with *Bacillus* spp. may provide benefits for ruminal fermentation characteristics, milk production efficiency, and cow health (Luan et al., 2015). Also, *B. licheniformis* has several practical advantages on facile cultivation and low-cost to produce feed additives (Wang et al., 2016). Taken together, we proposed that *B. licheniformis* and lichenicin 146 is capable for applying on the dairy field in safety respect as well as productive and economics.

In addition, RNA-Seq was performed to explore the gene regulation involved in the mode of action of lichenicin 146 against *Staph. aureus* strain RF122. Our transcriptome analysis showed that various genes were transcriptionally or post-transcriptionally regulated on treatment with lichenicin 146. In total, 23 genes were downregulated by more than 3-fold, whereas 23 genes

were upregulated (Table 1). In particular, the transcription levels of *SAB0133*, *SAB0357*, and *SAB2383*, which encode transcription regulators, dramatically increased by 220.5-, 39.8-, and 4.8-fold, respectively. According to these results, specific transcriptional regulators are involved in bacterial physiology, including cell viability (Balleza et al., 2009). In addition, genes encoding cell surface or membrane proteins (*SAB0993c*, *SAB0150*, *SAB0994c*, and *SAB2375c*) were strongly upregulated. In general, class II bacteriocins primarily act by making the membranes of target cells permeable, probably via pore formation (Ennahar et al., 2000). Similar results were reported by Martínez et al. (2007), who found that several membrane protein-encoding genes were significantly upregulated after the treatment of target cells with a bacteriocin. Overall, our results suggest that lichenicin 146 strongly affects the physiology of the cell membrane as well as the network of transcriptional regulators in the cytoplasm of mastitis-causing *Staph. aureus* strain RF122.

In conclusion, we established a *C. elegans*-based screening platform to target mastitis-causing *Staph. aureus* as a direct in vivo tool for screening potential bacteriocins. Our in vivo platform is simple, rapid, cost-effective, and reliable, and we anticipate that it will facilitate the discovery of many more potential bacteriocin-producing bacteria to inhibit mastitis-causing pathogens, including antibiotic-resistant bacteria in the dairy field.

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