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Bacillus licheniformis Isolated from Korean Traditional Food Sources Enhances the Resistance of *Caenorhabditis elegans* to Infection by *Staphylococcus aureus*

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We investigated whether *Bacillus* spp., newly isolated from Korean traditional food resources, influence the resistance of hosts to foodborne pathogens, by using *Caenorhabditis elegans* as a surrogate host model. Initially, we selected 20 *Bacillus* spp. that possess antimicrobial activity against various foodborne pathogens, including *Staphylococcus aureus*. Among the selected strains, six strains of *Bacillus* spp. used in preconditioning significantly prolonged the survival of nematodes exposed to *S. aureus*. Based on 16S rRNA gene sequencing, all six strains were identified as *B. licheniformis*. Our findings suggest that preconditioning with *B. licheniformis* may modulate the host defense response against *S. aureus*.

Keywords: *Bacillus licheniformis*, Korean traditional foods, *Caenorhabditis elegans*, defense response, antimicrobial activity

Fermented soybean products with *Bacillus* spp. are indigenous to Asian and African countries, which have long traditional ties to this nutritious food source. The fermentation products derived from these foods are also known to act as anticarcinogenic agents and antioxidants [8]. Out of these health-promoting strains, *Bacillus* spp. strains, including *B. subtilis* and *B. licheniformis*, are known to play a major role in the soybean fermentation process [10]. In other words, the quality and functionality of fermented soybean products such as *doenjang*, *cheongkookjang*, *kochujang*, and *kanjang* are affected by microbes, the fermentation process, and input materials such as soybeans or grains [15]. Previous studies showed that after co-inoculation of *B. licheniformis* SCK 121057 and *B. cereus* at a ratio of 10 to 1 on the surface of cooked soybeans, the cell count of *B. cereus* had been dramatically reduced after 31 days of incubation [10]. Furthermore, recent studies showed that certain strains of *B. licheniformis* confer significant potential probiotic agents for food or feed additives [14]. It has been shown that *B. licheniformis* strains ameliorate the gut microbiota and increase the growth and immune response of *Macrobrachium rosenbergii* in laying hens [3, 13]. In this study, we isolated potentially health-promoting strains of *B. licheniformis* with antimicrobial activities from Korean traditional food sources, and evaluated their effect against various foodborne pathogens and host defense responses, using the nematode *Caenorhabditis elegans* as a simple *in vivo* animal model.

Bacillus spp. were isolated from Korean traditional foods, including *doenjang*, *cheongkookjang*, *kochujang*, and *kanjang* (Sunchang, Jeollabuk-Do, Korea). Additionally, *M. luteus*, *B. cereus*, and *S. aureus* were obtained from the Korea

Agricultural Culture Collection (KACC, Suwon, Korea) and employed as indicator strains for antimicrobial activity. All bacterial strains were subcultured in TSB medium and incubated at 30ºC for 18 h in a shaking incubator at 150 rpm. The *C. elegans* strains used in this study were CF512 *fer-15(b26)II;fem-1(hc17)IV* [9]. This *C. elegans* strain was routinely maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50, as previously described [2]. The antimicrobial activity of the supernatant was evaluated by the agar well diffusion method [12] with some modifications. The *C. elegans* killing assay was performed as previously described [9]. L4/young adult worms were placed on conditioning plates with *Bacillus* spp. at 25ºC for 24 h. Next, nematodes with preconditioning by *Bacillus* spp. were transferred to prepared *S. aureus* pathogen plates. After the worms were placed on the pathogen plates, they were incubated at 25°C and examined at 24 h intervals for 15 days for viability using an Olympus SZ40 dissecting microscope. The worms were sorted as alive or dead by applying a gentle touch with a platinum wire. *C. elegans* survival was examined using the Kaplan-Meier method, and differences were determined with the log-rank test (STATA6; STATA, College Station, TX, USA). In addition, the number of bacterial cells in the worm intestines was measured according to previously described methods, with slight modifications [9]. *E. coli* OP50 was used as a normal-feeding control under the same experimental conditions.

Among 300 strains isolated from Korean traditional foods, the cell-free supernatants of 20 potentially health-promoting *Bacillus* spp. showed specific antibacterial activities against gram-positive pathogens, including *Micrococcus luteus*, *B. cereus*, and *Staphylococcus aureus* (Table 1). More than half of the 20 isolates showed inhibitory activity against *M. luteus* KACC 2177 (16 strains), *M. luteus* KACC 1056 (13 strains), *B. cereus* KACC 11341 (16 strains), and *S. aureus* KACC 1916 (12 strains). As previously reported, some *B. licheniformis* strains have been reported to produce lichenicidin, a two-peptide lantibiotic with strong activity against a range of pathogenic microorganisms, including *Listeria monocytogenes*, methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, and

Table 1. Antimicrobial activity caused by *Bacillus* spp. using the agar well diffusion assay.

Strains	Presence of a zone of inhibition with indicator strain:								
	Micrococcus luteus KACC			*Bacillus cereus* KACC			*Staphylococcus aureus* KACC		
	2177	1056	3624	11341	40935	10004	3881	1621	1916
SRCM 137	++	+	++	++	-	-	+	-	++
SRCM 138	++	+	-	-	-	-	-	-	-
SRCM 139	++	+++	++	++	++	++	++	++	++
SRCM 140	++	+++	++	++	-	++	++	++	++
SRCM 141	+++	+++	+++	++	-	-	++	++	++
SRCM 142	++	+	++	++	-	++	-	++	++
SRCM 143	+++	++	++	+	-	-	+	-	-
SRCM 144	++	+	++	-	-	++	-	+++	++
SRCM 146	++	+	++	++	-	-	+	+++	++
SRCM 147	+	+	-	++	-	-	++	+	++
SRCM 148	+	+	-	++	-	-	+	+	++
SRCM 150	-	-	-	-	-	++	-	-	-
SRCM 151	-	-	-	-	-	+	-	-	-
SRCM 152	-	+	-	++	-	-	-	-	-
SRCM 153	-	-	-	-	-	++	-	-	-
SRCM 155	+	-	++	+++	++	-	+	-	++
SRCM 156	+	-	-	+++	++	-	-	-	++
SRCM 158	+	-	-	++	-	-	-	-	-
SRCM 159	-	-	-	++	++	++	-	-	-
SRCM 160	+	+	++	+++	-	-	+	-	++

Diameter of the inhibition zone: (+) weak (6–9 mm), (++) intermediate (10–14 mm), (+++) strong (≥15 mm), (-) no inhibition zone.

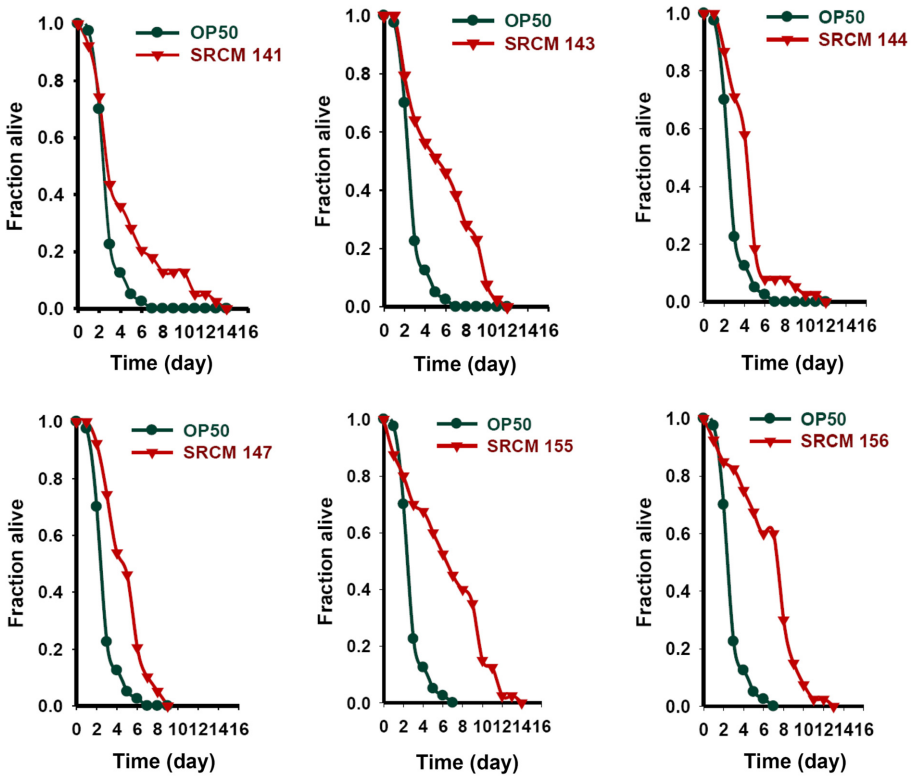

Fig. 1. Conditioning with the six *B. licheniformis* strains prolonged the survival of *C. elegans* nematodes infected with *S. aureus*. Survival statistics: all $p < 0.0001$ for SRCM 141-, 143-, 144-, 147-, 155-, and 156-conditioned nematodes compared with worms feeding on the *E. coli* OP50 control strain.

B. cereus [1]. Moreover, recent research was published demonstrating that the culture supernatant of the newly isolated *B. licheniformis* showed inhibition of both gram-positive and gram-negative bacteria [5]. Hence, we concluded that the 20 isolates of *Bacillus* spp. from Korean traditional foods have strong antimicrobial activity against foodborne pathogens, especially gram-positive organisms.

Importantly, specific antimicrobial molecules may facilitate the introduction of a producer into an established niche, directly inhibit the invasion of competing strains or pathogens, or modulate the composition of the microbiota and influence the host immune system [4]. Some *B. licheniformis* strains have been reported to stimulate the immune response of chickens [11]. Therefore, we explored whether *Bacillus* spp. that possess antimicrobial activity can augment the host defense response to *S. aureus*. Here, the nematode *C. elegans* (*fer-15;fem-1*) was used as a simple *in vivo* defense response model. *C. elegans* has been accepted as an alternative model host for the study of microbial infection and is a simple animal model with which it is possible to study evolutionarily conserved aspects of innate immunity [9]. In this study, we used an agar-based solid killing assay. Worms were conditioned by transferring young adult/L4 worms to each bacilli lawn for 24 h and then transferring them to *S. aureus* using solid killing assays with *fer-15;fem-1* worms. The conditions with each *Bacillus* spp. for 24 h did not affect any of the physiological characteristics of *C. elegans*, including body and brood size, compared with those of worms given the *E. coli* OP50 control (data not shown). Remarkably, the viability of *C. elegans* was significantly enhanced or similar to that of control worms that were pre-exposed to the nonpathogenic *E. coli* strain OP50 (the standard laboratory food source for *C. elegans*) when nematodes were conditioned on lawns of *Bacillus* spp., and then exposed to an *S. aureus* infection under the solid killing conditions (Fig. 1). In particular, among the 20 isolates of *Bacillus* spp., SRCM 141, 143, 144, 147, 155, and 156 dramatically enhanced the viability of *C. elegans* infected with *S. aureus* ($p < 0.0001$; Fig. 1). Conversely, *B. licheniformis* SRCM 153 and 158 negatively influenced the extension of the lifespan of the *C. elegans* host (data not shown). Taken together, we

concluded that conditioning with *Bacillus* spp. SRCM 141, 143, 144, 147, 155, or 156 makes *C. elegans* worms resistant to infection by *S. aureus*.

Next, we evaluated the number of *Bacillus* spp. in the *C. elegans* intestinal tract. Unexpectedly, the number of *Bacillus* spp. cells attached to the intestinal epithelial cells was similar with those of *E. coli* OP50, which did not enhance the resistance of *C. elegans* to *S. aureus* infection (data not shown). Therefore, we suggest that there are no critical correlations between the level of bacilli colonization and the defense responses in the *C. elegans* intestine. In other words, these data indicated that the number of colonized cells do not impact the defense response directly. One possibility is that when intact bacilli cells are digested by the worm grinder, specific microbial components (*e.g.*, cell wall molecules or bacteriocins) may influence the host defense responses. Finally, 16S rRNA sequencing showed that the six host defense-enhancing *Bacillus* spp. were identified as *B. licheniformis*. Consistent with a previous report [6], our findings demonstrate that *B. licheniformis* can be employed for a range of different biotechnological applications, such as dietary supplements for humans and animal feed inoculants, owing to their ability to stimulate the host defense system. Recently, our group reported that *B. licheniformis* activates key immune signaling pathways involved in *C. elegans* defenses, including *pmk-1* [7], which encodes the p38 mitogen-activated protein kinase pathway (2014 General Meeting of American Society for Microbiology, Boston, MA; No. 2271). An ongoing study is evaluating the specific host defense mechanism of *C. elegans via B. licheniformis* using whole-transcriptome analysis.

In conclusion, we showed that preconditioning with *B. licheniformis* SRCM 141, 143, 144, 147, 155, and 156 isolated from Korean traditional foods significantly stimulates *C. elegans* host defenses, independently of their level of colonization in the worm intestine. Our results suggest the potential application of the six newly isolated *B. licheniformis* strains in functional foods/feeds and therapeutic dietary supplements.

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