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Comparing the mortality of *Protaetia brevitarsis seulensis* (Coleoptera: Cetoniidae) caused by entomopathogenic bacteria and *Serratia marcescens* (Enterobacteriales: Enterobacteriaceae)

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Abstract

To investigate whether *Serratia marcescens* (Enterobacteriales: Enterobacteriaceae) isolated from *Protaetia brevitarsis seulensis*(Coleoptera: Cetoniidae) acts as an opportunistic bacterium in peroral infection, the primary entomopathogenic bacteria *Bacillus thuringiensis* (Bacillales: Bacillaceae) and *Paenibacillus popilliae* (Eubacteriales: Bacillaceae) were added to sawdust to perform a bioassay experiment. We found that peroral infection caused by *S. marcescens* could be fatal beyond a concentration of 4×10^8 pfu/mL in 2nd stage *P. b. seulensis* larvae and at 6×10^8 pfu/mL in 3rd stage *P. b. seulensis* larvae. In particular, mortality resulting from a combination of *P. popilliae* and *S. marcescens* was markedly increased in 2nd stage *P. b. seulensis* larvae. Therefore, we confirmed that mortality was increased when *S. marcescens* was infected together with other entomopathogenic bacteria, and that peroral infection itself can be fatal beyond certain concentrations.

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Bioassay,
Mortality

Introduction

Recently, several studies investigating insect biological resources have been performed in Korea. Some of these cases involved insects that are used as traditional medicines with proven efficacy (Chung *et al*. 2013a; Chung *et al*. 2013b; Kwon *et al*. 2013). The distribution and sales of certain edible insects have accelerated the industrialization of insects; however, the mass breeding of insects has also caused an increase in diseases (Kwak *et al*. 2014; Lee *et al*. 2015). Accordingly, increased monitoring has been initiated for diseases occurring in farms of *Protaetia brevitarsis seulensis* (Coleoptera: Cetoniidae), an edible insect that is mass-reared in Korea, as well as the Japanese rhinoceros beetle *Allomyrina dichotoma* (Coleoptera: Scarabaeidae), and diseases with a high incidence were identified. Insects are mainly infected by fungi, bacteria, viruses, and protozoa, and mostly show cross-infection of fungi-bacteria, fungi-viruses, or fungi-bacteria-viruses. Statistical analysis of entomopathogenic microorganisms discovered at *P. b. seulensis* farms from February 2013 to July 2014 showed that the most common bacterium was *Serratia marcescens* (Enterobacteriales: Enterobacteriaceae), with an infection rate of 60% revealed by

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polymerase chain reaction. Furthermore, there was no mortality observed in 3rd stage larvae after 4 wk of peroral infection due to intake of *S. marcescens*. However, when a peroral infection experiment was performed at an elevated bacterium concentration of 1×10^8 /mL in 2nd stage *P. b. seulensis* larvae, *S. marcescens* was found to be fatal.

S. marcescens is an entomopathogenic bacterium that is commonly used as an insecticide (Carol R. Lauzon 2003; Ishii *et al*. 2014). Several investigations have shown that *S. marcescens* is lethal to many kinds of insects, including *Bombyx mori* (Lepidoptera: Bombycidae), *P. b. seulensis* (Coleoptera: Cetoniidae), and *Rhagoletis pomonella* (Diptera: Tephritidae) (Ishii *et al*. 2014; Kwak *et al*. 2014; Lauzon C. R. 2003). Moreover, *S. marcescens* is also considered to be an opportunistic pathogen and can cause antibiotic-resistant hospital-acquired infections (Murdoch *et al*. 2011). *S. marcescens* can outcompete other bacteria and survive to express antibacterial-resistant activity (Murdoch *et al*. 2011). *Paenibacillus popilliae* (Eubacteriales: Bacillaceae) causes milky disease and is known as an obligate pathogen in Japanese beetle and other scarab larvae (F. E. El-Borai 2005; Harrison *et al*. 2000). In addition to *P. popilliae,* a novel *Paenibacillus sp*. was found to be an obligate entomological pathogen (F. E. El-Borai 2005). *Bacillus thuringiensis* (Bacillales: Bacillaceae) is a soil bacterium that has been used as a safe and environmentally friendly biopesticide (Gao *et al*. 2012), and its main toxins include Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa (Noguera and Ibarra 2010; Stalinski *et al*. 2014). In particular, the Cry and Cyt toxins are proteins that are used worldwide for insect control, and have been shown to interact with receptors on the host cell surface (Bravo *et al*. 2007; Stalinski *et al*. 2014).

Materials and Methods

Insect rearing

P. b. seulensis (Kolbe) was reared in the plastic cages (60 × 33 × 38 cm) at 25°C, 40–50% relative humidity with autoclaved sawdust. For the experiments, only 2nd and 3rd stage larvae of *P. b. seulensis* were collected. The insects were starved for 24 h before entomopathogen treatment.

Bacteria

Bacillus thuringiensis (KACC10169) and *Serratia marcescens* (KACC11892) were purchased from the Korean Agricultural Culture Collection (KACC, Wanju, Korea). *Paenibacillus popilliae* (KCTC3806) was purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). Lyophilized bacteria were incubated on nutrient agar plates, grown for 18–24 h, and colonies were isolated. The cultured bacteria were diluted to a total concentration of 1×10^8 pfu/mL using a hemocytometer.

Entomopathogen application and observation

We placed seven 2nd stage *P. b. seulensis* larvae onto separate plates 4.5 cm in diameter and 1 cm in height. We treated 2 g of fermented sawdust per insect with 4 mL (4×10^8 pfu/mL) of each bacterium: *B. thuringiensis* (Bt), *P. popilliae* (Pp) and *S. marcescens* (Sm). The bacteria mixtures (Bt+Sm, Pp+Sm) were prepared with 2 mL Bt, 2 mL Sm, or 2 mL Pp, depending on the combination, to achieve the

Table 1. Material and rate of application for each treatment for 2nd stage larvae.

Treatment	Source	Material	Rate (active ingredient)
C	N/A	Control (Distilled Water)	4 mL/1plate[1]
NB	N/A	Autoclaved Nutrient Broth	4 mL/1plate[1]
Bt	KACC10169	*B.thuringiensis*	4mL/1plate[1]
Bt+Sm	KACC10169 + KACC11892	*B.thuringiensis+S.marcescens*	2 mL/plate[1]+2mL/plate[1]
Pp	KCTC3806	*P.popilliae*	4 mL/plate[1]
Pp+Sm	KCTC3806 + KACC11892	*P.popilliae+S.marcescens*	2 mL/plate[1]+2mL/plate[1]
Sm	KACC11892	*S.marcescens*	4mL/plate[1]

[1]Platesize:area;r=4.5cm, h = 1 cm

Table 2. Material and rate of application in each treatment for 3rd stage larvae.

Treatment	Source	Material	Rate (active ingredient)
C	N/A	Control (distilled water)	6 mL/plate[1]
NB	N/A	Autoclaved nutrient broth	6 mL/plate[1]
Bt	KACC10169	*B.thuringiensis*	6mL/plate[1]
Bt+Sm	KACC10169 + KACC11892	*B.thuringiensis*+*S.marcescens*	3 mL/plate + 3 mL/plate[1]
Pp	KCTC3806	*P.popilliae*	6 mL/1plate[1]
Pp+Sm	KCTC3806 + KACC11892	*P.popilliae*+*S.marcescens*	3 mL/plate + 3 mL/plate[1]
Sm	KACC11892	*S.marcescens*	6 mL/plate[1]

Plate[2] size:area;r=4.5cm,h=3cm

same total bacterial concentration (Table 1). Similarly, we placed 3rd stage *P.b. seulensis* larvae individually onto plates 4.5 cm in diameter and 3 cm in height, and treated 4 g of fermented sawdust per insect with 6 mL (6×10^8 pfu/mL) of each bacterium (Bt, Pp, Sm). The bacteria mixtures (Bt+Sm, Pp+Sm) were prepared with 3 mL Bt, 3 mL Sm, or 3 mL Pp to achieve the same total bacterial concentration (Table 2). We used the same volumes of distilled water as a negative control, and the same volumes of the nutrient broth from the bacterial culture as a positive control. The experiment was repeated three times, and the *P. b. seulensis* treated with bacteria were examined for 4 wk.

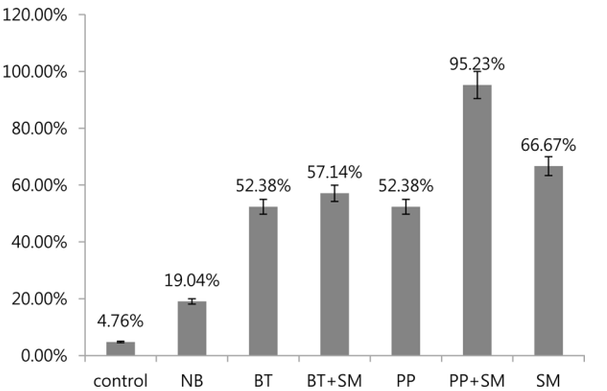

Fig. 1. Mortality rate (%) of 2nd stage *Protaetia brevitarsis seulensis* larvae treated with different combinations of bacteria. C, water (negative control); NB, positive control (nutrient broth); BT *Bacillus thuringiensis*; BT+SM, *Bacillus thuringiensis* + *Serratia marcescens*; PP, *Paenibacillus popilliae*; PP+SM, *Paenibacillus popilliae* + *Serratia marcescens;* SM, *Serratia marcescens.*

Results and Discussion

A previous study showed high mortality of 3rd stage *P. b. seulensis* larvae via hemolymph injection of *S. marcescens* (Kwak *et al*. 2014), but showed 0% mortality when 3rd stage *P. b. seulensis* larvae ingested *S. marcescens* (1×10^2, 1×10^4, 1×10^7 pfu/mL) together with sawdust. In this study, we investigated the mortality rate resulting from cross-infection with *S. marcescens* and entomopathogenic bacteria (*B. thuringiensis* and *P. popilliae*) that occur commonly in *P. b. seulensis* with a high fatality rate. We also confirmed the effect of *S. marcescens* on mortality, which is diagnosed very frequently in *P. b. seulensis* breeding farms and is known as an opportunistic bacterium. The results of this study confirmed that mortality increased in insects with peroral infections of *S. marcescens*, similar to other bacteria. We observed mortality after 4 wk when insect sawdust was treated with Sm together with Bt and Pp, which are known to be pathogenic in Coleoptera. In 2nd stage *P. b. seulensis* larvae, Bt treatment at 4×10^8 pfu/mL per insect resulted in 52.38% mortality. The Bt+Sm combination treatment resulted in 57.14% mortality, which was 4.74% higher than Bt treatment alone. Pp treatment resulted in 52.38% mortality, whereas the combination of Pp+Sm resulted in a mortality rate of 95.23%, which was 42.85% higher than Pp treatment alone (Fig. 1). Sm treatment resulted in a 14.29% higher mortality rate than Bt and Pp.

In 3rd stage *P. b. seulensis* larvae, Bt treatment at 6×10^8 pfu/mL per insect resulted in 38.04% mortality, and the Bt+Sm combination resulted in a mortality rate of 28.61%. Pp treatment resulted in 33.33% mortality, whereas mortality for the Pp+Sm combination was 38.09%, which was 4.76% higher. Sm treatment resulted in a rate of 23.80%, which was lower than

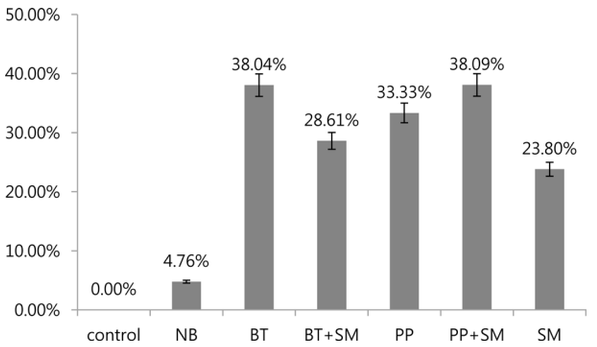

Fig. 2. Mortality rate (%) of 3rd stage *Protaetia brevitarsis seulensis* larvae treated with different combinations of bacteria. C, water (negative control); NB, positive control (nutrient broth); BT *Bacillus thuringiensis*; BT+SM, *Bacillus thuringiensis* + *Serratia marcescens*; PP, *Paenibacillus popilliae*; PP+SM, *Paenibacillus popilliae* + *Serratia marcescens;* SM, *Serratia marcescens.*

those of Bt and Pp. These results confirmed that mortality is dependent on the insect developmental stage. In 2nd stage larvae, mortality due to *S. marcescens* was higher than that caused by *B. thuringiensis* and *P. popilliae*, whereas it was lower in 3rd stage larvae (Fig. 2). In other words, *S. marcescens* induced stronger mortality in 2nd stage larvae than 3rd stage larvae, and showed mortality with higher-efficiency in 2nd stage larvae compared to *B. thuringiensis* and *P. popilliae*. The Pp+Sm combination treatment revealed a very strong synergistic effect in 2nd stage *P. b. seulensis* larvae compared to the Pp treatment alone, and its mortality rate after 4 wk was 95.23%, which was 42.85% higher than the Pp treatment alone. Since mortality is maximal when *P. popilliae* and *S. marcescens* are detected together in a breeding environment or in sawdust, the results of this study can be utilized in the prevention and diagnosis of such cases. *S. marcescens*, an entomopathogen, has been reported to be an opportunistic bacterium (Murdoch *et al*. 2011), but the results of this study confirmed that it can also show higher mortality than *B. thuringiensis* and *P. popilliae*, beyond a certain concentration, which proves that its fatal effects are stronger at younger larval stages. Furthermore, this study provides valuable information with respect to predicting the effect of these bacteria on mortality when they are detected together at insect breeding sites, which will make prevention possible. In future studies, it will be necessary to investigate the mechanisms of how *S. marcescens* interacts with *P. popilliae* to maximize mortality and to examine the specific mortality rate of each bacterium for each stage of insect development.

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