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Growth-dependent toxicity of *Photorhabdus temperata* in katydid *Paratlanticus ussuriensis*

Jae-Kyoung Shim [a], Hea-Son Bang [b], Kyeong-Yeoll Lee [a,*]

[a] Division of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu, 702-701, Republic of Korea
[b] National Institute of Science and Technology, RDA, Suwon 441-707, Republic of Korea

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ABSTRACT

The oral toxicity of the symbiotic bacteria *Photorhabdus temperata* was investigated in various developmental stages of *Paratlanticus ussuriensis*. Supernatants of *Photorhabdus* culture medium were mixed into an artificial diet, which was fed to various stages of immature nymphs and adults of *P. ussuriensis*. Mortality was highest in the first instar nymph but decreased in older stages of immature nymphs. Adult females were not killed upon oral ingestion of *P. ussuriensis*, but their fecundity was significantly inhibited to 29.3% of that of control. In addition, the effects of oral ingestion of the symbiont culture media on the expression rates of three heat shock protein 70 genes (*hsp70a*, *hsp70b*, and *hsp70c*) in third instar nymphs of *P. ussuriensis* were determined by quantitative real-time RT-PCR analysis. There were no significant changes in expression levels in comparison with control, which suggests that hsp genes may not be associated with the mechanism of *Photorhabdus* toxicity. Our results imply that *Photorhabdus* culture media is highly effective in killing younger immature nymphs and also suppressing adult reproduction of *P. ussuriensis*.

Introduction

Photorhabdus is a Gram-negative bacterium that symbiotically resides in the gut of soil-dwelling entomopathogenic nematodes of the genus *Heterorhabditis* (Forst et al., 1997; ffrench-Constant et al., 2007). The high toxicity of entomopathogenic nematodes is due to insecticidal factors, which are synthesized and released by symbiotic bacteria into the hemolymph of target insects (Forst et al., 1997; Kim et al., 2005; ffrench-Constant et al., 2007; Shrestha and Kim, 2010; Shrestha et al., 2011). The overall insecticidal toxicity of *Photorhabdus* is caused by a combination of toxic factors, including a mixture of both proteins and metabolites. Many of these factors are pathogenic upon either injection into the hemolymph or oral ingestion (ffrench-Constant et al., 2007). Among them, the most well known toxin is the toxin complex (Tc) from *P. luminescens*, which consists of at least 10 polypeptides grouped into three functional components (A, B, and C) (Bowen et al., 1998). Additionally present are 'Photorhabdus insect-related' (Pir) toxins consisting of binary toxins PirAB (Duchaud et al., 2003). Both Tc and Pir toxins are pathogenic upon oral administration as well as hemolymph injection into insects. Other toxins include 'make caterpillars floppy' (Mcf) and proteins encoded by the 'Photorhabdus virulence cassettes' (PVCs), which only show injectable activity into the hemolymph (Daborn et al., 2002). Recently, numerous factors have been identified as pathogenic, and their genetic and molecular information have been determined. However, the practical application of pathogenic factors for pest control in agricultural fields remains elusive.

Practical applications of various formulations of entomopathogenic nematodes have been conducted for pest control both under field and greenhouse conditions (Gaugler and Kaya, 1990; Kim et al., 2008). However, their control efficacy has remained low due to the damage induced by dehydration and UV radiation (Smith, 1996). However, recent developments in microbe culture technology have allowed the mass production of bacterial formulations of *Photorhabdus* isolated from entomopathogenic nematodes for the purpose of pest control (Wang et al., 2007). Specifically, field application of bacterial formulations is more convenient and can also improve control efficacy as opposed to nematode formulations.

The katydid *Paratlanticus ussuriensis* has become overpopulated in the mountain area of Yeongdong County, Korea from 2006 to several consecutive years (Na et al., 2007; Bang et al., 2008; Moon et al., 2009; Jung et al., 2011). *P. ussuriensis* seriously damages horticultural crops such as apples, peaches, and pears, which are cultivated on the hillsides of mountains. Various synthetic pesticides have been tested to control this species, but their use could be harmful to many other non-target insects that habitat mountain areas (Ahn et al., 2007). Currently, several control techniques featuring natural attractants and LED light have been applied in an environment-friendly manner for control of *P. ussuriensis* (Nho et al., 2008; Jung et al., 2009). Here, we investigated whether or not the toxicity of *P. temperata* culture media can be used as a biopesticide for the control of *P. ussuriensis*. Toxicity of *P. temperata* was determined by analyzing the mortality levels of various stages of

* Corresponding author. Tel.: +82 53 950 5759; fax: +82 53 950 6758.
E-mail address: leeky@knu.ac.kr (K.-Y. Lee).

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Table 1
Primer information for real-time PCR analysis of *Paratlanticus ussuriensis*.

Target gene	Primer	Sequence (5′ → 3′)	Length (bases)	Product (bp)
hsp70a	forward	ATGGCTTGACAGCAATGCTCTT	22	71
	reverse	ACTTGCTTTGCAATTCTTTCAACTTGT	27	
hsp70b	forward	TTCACGGACACGGAGCG	17	77
	reverse	TCAAACACCGTGTTCTTCGG	20	
hsp70c	forward	ACAGCCGGCGACACTCA	17	56
	reverse	TGATTTACCAGACGGCTATCGA	22	
16S rRNA	forward	CCGTGCAAAGGTAGCATAATCA	22	66
	reverse	TGCCTCGTCAAACCATTCATT	21	

immature nymphs as well as the fecundity of female adults. In addition, transcriptional changes of three heat shock protein (hsp) genes were measured in order to understand the physiological response of *P. ussuriensis* to toxic factors of *P. temperata*.

Materials and methods

Insects

Eggs that experienced two warm-chill cycles, which induced diapause termination, were incubated at room temperature as described previously (Bang et al., 2011). Hatched nymphs were reared individually at 25 ± 1 °C, 40–60% relative humidity (RH), and under a 14:10 h light:dark (L:D) photoperiod. An artificial diet consisting of wheat bran and fishmeal (1:1) in water was supplied every 2 days. Each developmental stage of the immature nymphs and adults was observed and body sizes were measured (Moon et al., 2009). Female adults oviposited eggs in a mixture of soil and vermiculite (1:1) (Bang et al., 2009).

Supernatant isolation from culture medium of Photorhabdus temperata

The symbiotic bacterium *P. temperata* was cultured in nutrient bromothymol blue agar (NBTA) medium for 72 h as described in Shrestha et al. (2011). Bacterial culture medium (3.5×10^7 cfu/ml) was centrifuged at 10,000 g for 20 min at 4 °C. Supernatant was transferred to new plastic tubes and stored at 4 °C until used.

Ingestion of the supernatant of culture medium of Photorhabdus temperata

Nymphs and adults were reared individually in a plastic cage. They were allowed to ingest an artificial diet mixed at a 1:1 ratio (*w*/*v*) in the supernatant of *Photorhabdus* culture medium. Treated katydids ($n = 20$ in each group) were kept in an incubator at 25 °C. Each set of experiments was conducted three times on different dates at the conditions of 25 ± 1 °C, 40–60% RH.

Quantitative real-time RT-PCR (qRT-PCR) analysis

Total RNA was isolated from whole bodies of third instar nymphs using an RNeasy mini kit (Qiagen, USA). The cDNAs were synthesized with total RNA (1 μg) using a Reverse Transcriptase System Kit (Applied Biosystems, USA). Using nucleotide sequences from the NCBI database, gene-specific primers were designed for quantitative real-time PCR (Table 1). PCR amplifications were performed in a 25 μl reaction containing cDNAs (0.2 μl) generated from 2 μg of the original RNA template, 80 nM each of the gene-specific primers, and 12.5 μl of Power SYBER Green Master Mix (Applied Biosystems). The amplified signals were monitored continuously with the 7300 Sequence Detection System (Applied Biosystems), and the amplification protocol was as follows: 1 cycle of denaturation (95 °C for 10 min), 40 cycles of extension (95 °C for 15 sec; 55 °C for 20 sec; 72 °C for 35 sec), and 1 cycle for dissociation (95 °C for 15 sec; 60 °C for 30 sec; 95 °C for 15 sec). The threshold cycle (Ct) values were used to calculate the relative quantities of *hsp70a* (JF966356), *hap70b* (KC206085), *hsp70c* (JX112893) and *16S rRNA* (KC251735). The level of *16S rRNA* was used as a reference to normalize the expression levels between samples. All data were relatively calculated and expressed against *16S rRNA* levels in order to compensate for any differences in reverse transcriptase efficiency. Experimental data of mRNA levels of *hsp70* were subjected to analysis of variance (ANOVA) using an SAS program (2000).

Statistical analysis

Mortality rates and mRNA levels were reported as mean ± standard error. Analysis of variance (ANOVA) was carried out to analyze the means using a proc general linear model (GLM) with Statistical Analysis System program (SAS, 2003) version 9.1 to identify significant effects of dose-specific treatments. Separation of treatment means was conducted by using Duncan's multiple range tests at the 95% confidence level. Data were analyzed by completely randomized design with three replications.

Results

Oral toxicity of Photorhabdus culture media in immature and adult stages of P. ussuriensis

To determine the oral toxicity of *Photorhabdus* culture broths in immature stages of *P. ussuriensis*, various stages of nymphs were allowed to ingest an artificial diet containing supernatants of *P. temperata*. Mortalities of the control groups that ingested only the artificial diet were in the range of 12.5% to 23.2% during the test rearing period of either 16 or 25 days, whereas those of treated groups were 93.3%, 65.0%, 36.6%, and 21.9% at 1st, 2nd, 3rd, and both 4th and 5th instars, respectively (Table 2). Thus, the *Photorhabdus* culture medium was highly toxic to younger instars but less or not toxic to older immature stages.

Table 2
Effects of oral ingestion of *Photorhabdus* culture supernatant on mortality levels of various developmental stages of *Paratlanticus ussuriensis*.

Instars	Treatments	Mortality (% ± SE)					
		Day 1	Day 4	Day 7	Day 10	Day 13	Day 16
1st	Control	6.7 ± 0.0^a	16.7 ± 3.4^a	16.7 ± 3.4^b	16.7 ± 3.4^b	20.0 ± 0.0^b	20.0 ± 0.0^b
	Pt-treated	3.4 ± 3.6^a	10 ± 3.3^a	66.7 ± 6.7^a	83.4 ± 3.4^a	83.4 ± 3.4^a	93.3 ± 0.0^a
2nd	Control	0.0 ± 0.0^a	2.5 ± 2.5^a	7.5 ± 2.5^a	10.0 ± 5.0^b	12.5 ± 2.5^b	12.5 ± 2.5^b
	Pt-treated	0.0 ± 0.0^a	7.5 ± 2.5^a	10.0 ± 5.0^a	37.5 ± 2.5^a	57.5 ± 7.5^a	65.0 ± 10.0^a
		Day 1	Day 5	Day 10	Day 15	Day 20	Day 25
3rd	Control	0.0 ± 0.0^a	5.1 ± 2.6^a	5.1 ± 2.6^a	9.7 ± 2.3^a	17.1 ± 2.3^b	17.1 ± 2.3^b
	Pt-treated	0.0 ± 0.0^a	0.0 ± 0.0^a	0.0 ± 0.0^a	5.5 ± 2.8^a	36.6 ± 0.2^a	36.6 ± 0.2^a
4, 5th	Control	0.0 ± 0.0^a	0.0 ± 0.0^a	0.0 ± 0.0^a	19.0 ± 4.3^a	23.2 ± 3.0^a	23.2 ± 3.0^a
	Pt-treated	0.0 ± 0.0^a	0.0 ± 0.0^a	4.2 ± 2.4^a	17.4 ± 0.4^a	21.9 ± 3.1^a	21.9 ± 3.1^a

Means within a column followed by the same small letter do not differ significantly according to DMRT at 5% level of significance. These data represent mortality percentages of young stages of *Paratlanticus ussuriensis* when exposed to experimental diet for 25 days.