[Short Communication]

The insecticidal activity of an entomopathogenic nematode *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens* against *Periplaneta* and *Blattella* cockroaches

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Photorhabdus luminescens is a gram-negative luminescent enterobacterium that symbiotically resides in the digestive tract of an entomopathogenic nematode (EPN) of the family Heterorhabditidae. *Heterorhabditis bacteriophora* upon entering an insect host, regurgitate *P. luminescens* cells, and the bacteria quickly establish a lethal septicemia. To expand the possibility of its cockroach control, the insecticidal activity of this nematode-bacterium complex against cockroaches was investigated in two ways. First, we examined the insecticidal activity of *H. bacteriophora* harboring *P. luminescens* against the middle-stage instar of three *Periplaneta* (*P. fuliginosa*, *P. americana*, and *P. japonica*) and one *Blattella* (*B. germanica*) cockroach species. Mortality of *P. fuliginosa* and *P. americana* were significantly increased when treated with 20,000 and 100,000 nematodes/0.3 g bait/ 5 cockroaches. The mortality of *P. japonica*, however, did not differ from that of the control at all of the tested concentrations; whereas, the mortality of *B. germanica* was relatively higher than that of *Periplaneta* cockroaches. Second, we tested the pathogenicity of *P. luminescens* alone against the three *Periplaneta* species by injection of bacterial cells. All tested cockroaches were found to be killed within 2–4 days. These results would suggest that *Periplaneta* are susceptible to *P. luminescens*; however, in the bait experiment, *H. bacteriophora* was unable to effectively establish a lethal infection of *P. luminescens*. Our results indicate that susceptibility to EPN infection and bacterial pathogenicity should be evaluated separately,

and that much less *H. bacteriophora* is required to kill *B. germanica* than is needed to kill three *Periplaneta* cockroaches. Nematol. Res. 50(2), 27–33. (2020)

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INTRODUCTION

The entomopathogenic nematodes (EPNs) are a group of insect-killing nematodes in two genera, *Heterorhabditis* and *Steinernema*, that are respectively associated symbiotically with the entomopathogenic bacteria *Photorhabdus* and *Xenorhabdus*. Due to their high pathogenicity and insect-seeking ability, EPNs have been developed as biological control agents for agricultural and urban pests (Appel *et al.*, 1993; Shapiro-Ilan *et al.*, 2002; Dolinski *et al.*, 2012). Infective juveniles (IJs), a special stage of EPNs, sense several cues (*e.g.*, volatiles such as CO₂) from the insect host (Hallem *et al.*, 2011; Dillman *et al.*, 2012), forage, and invade the insect body from the anus, mouth, or spiracle, whereby releasing their symbiotic bacteria into the insect hemocoel (Ciche and Ensign, 2003; Ciche, 2007). These bacteria suppress insect host defenses such as phenoloxidase (Eleftherianos *et al.*, 2007) and produce insecticidal toxins to kill insect hosts within 72 h (Dowds and Peters, 2002; Forst and Clarke, 2002). EPNs continue to develop and propagate by feeding on the symbiotic bacteria until food is depleted and there is a high nematode density in the insect cadaver. Then, IJs, which are re-associated with the bacteria, escape from the cadaver to seek a new host (Ciche, 2007).

Cockroaches belonging to the order Blattodea are a highly diverse group of insects comprising over 4,000 species in 460 genera worldwide (Rentz, 2014). Cockroach species such as *Periplaneta fuliginosa*, *P. americana*, and *Blattella germanica* have extremely high environmental adaptability, and are now spreading and inhabiting many areas of the world through modern human economic activities (Appel and Smith, 2002; Miller and Meek, 2004; Kramer and Brenner, 2009; The Japanese Society of Pestology, 2015). Cockroaches can transmit diverse human pathogens that result in serious human disease epidemics (Pan and Zhang, 2020), and thus they are considered to be one of the most serious urban sanitary pests.

To expand the possibility of using EPNs in pest control strategies, the susceptibilities of various insect hosts to EPNs have been tested (Zioni Cohen-Nissan *et al.*, 1992; Rahimi *et al.*, 1993; Hashmi *et al.*, 1995;

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O'Leary and Burnell, 1997; Ciche and Sternberg, 2007; Moshayov *et al.*, 2013; Bai *et al.*, 2013), including cockroaches infected with *Heterorhabditis* (Zervos and Webster, 1989; Maketon *et al.*, 2010; Baker *et al.*, 2012), and *Steinernema* (Corpus and Sikorowski, 1992; Koehler *et al.*, 1992; Appel *et al.*, 1993; Appel and Benson, 1994; Maketon *et al.*, 2010; Baker *et al.*, 2012; Morton and García-del-Pino, 2013). These studies, however, only focused on the mortality of cockroaches exposed to EPNs. Because the pathogenicity of EPNs, especially those in the genus *Heterorhabditis*, largely depends on the pathogenicity of the associated bacteria (Han and Ehlers, 2000), it is also important to evaluate the insecticidal activity of these bacteria separately from that of EPNs. To expand the possibility of using EPNs for cockroach control, this report describes the insecticidal activity of *Heterorhabditis bacteriophora* harboring *P. luminescens*, *i.e.*, the nematode-bacterium complex, and *P. luminescens* alone, against three *Periplaneta* and one *Blattella* cockroach species, which are well-known sanitary pests in Japan.

MATERIALS AND METHODS

Insects and nematodes:

Four cockroach species were used in these experiments: *P. fuliginosa* EE, *P. americana* NC, *P. japonica* Miyoshi (Ozawa *et al.*, 2016; Ozawa and Hasegawa, 2018), and *B. germanica* Iga. Dozens of *B. germanica* were collected directly by hand in Iga City, Mie Prefecture, Japan, in November 2014, and have been maintained in the Hasegawa Laboratory (Chubu University, Japan) and established as the *B. germanica* Iga strain. These four species were reared following the method described by Ozawa *et al.* (2014). *H. bacteriophora* TT01 harboring its mutualistic bacterium *P. luminescens* TT01 (kindly provided by Ann Burnell, National University of Ireland-Maynooth) was maintained by infecting the greater wax moth *Galleria mellonella* larvae as described previously (Kaya and Stock, 1997). *P. luminescens* was isolated from *G. mellonella* infected with *H. bacteriophora* as described previously (Sato *et al.*, 2014).

EPN pathogenicity against cockroaches:

The middle-stage instar cockroaches were fasted for three days before the infection experiments. Five fasted cockroaches per each group were reared in a plastic pack (117.5 × 117.5 × 85.5 mm) with 0.3 g of powdered bait (Ozawa *et al.*, 2014) mixed with the

fresh infection stage of *H. bacteriophora* (0, 2,000, 20,000, and 100,000 nematodes in 1 ml of sterilized distilled water) for two weeks at room temperature (about 25°C). After this initial feeding, 0.3 g of fresh bait without nematodes was added every three days. The survival of cockroaches was checked every two days. Dead cockroaches were transferred individually onto a filter paper moistened with sterilized distilled water in a 6-cm petri dish, sealed with parafilm, and incubated for one week at 25°C. One week later, the cockroach carcasses were dissected and EPN infection was checked. Cockroaches that had died, but not due to EPN infection, were excluded from the analysis. The experiments were repeated three to six times. Survival curves were analyzed using the Kaplan–Meier procedure, and significant differences between survival curves were calculated by the log-rank test using EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda, 2013).

Bacterial pathogenicity against cockroaches:

Injection experiments against cockroaches were performed as described by Sato *et al.* (2016). Briefly, the mid-exponential phase of *P. luminescens* and *E. coli* OP50 (as a control) cultured in LB broth at 30°C or 37°C were washed with sterilized phosphate-buffered saline (PBS, 0.8 g NaCl, 0.02 g KCl, 0.14 g Na₂HPO₄, and 0.02 g KH₂PO₄ in 100 ml of DW) and re-suspended in sterilized PBS at the appropriate cell densities (7×10^8 cells/ml). This bacterial concentration was used in a previous study and confirmed to provide sufficient pathogenicity of *P. luminescens* against *Zophobas morio* (Sato *et al.*, 2016). The middle-stage instars of the *Periplaneta* cockroaches (*P. fuliginosa*, *P. americana*, *P. japonica*) were chilled on ice for a few minutes, and then 5 µl of bacterial suspension was injected into the body cavity of the abdomen by using a 1702RN Neuros syringe (Hamilton Co., NV, USA). Three individuals of injected cockroaches were reared in a plastic case at 25°C and observed every second day. The experiments were repeated three to five times independently. Survival curves were analyzed using the Kaplan–Meier procedure, and significant differences were calculated by the log-rank test using EZR software (Kanda, 2013).

RESULTS AND DISCUSSION

We investigated the survival of three *Periplaneta* and one *Blattella* cockroaches fed with various concentrations of bait containing *H. bacteriophora* to

reveal the insecticidal activity of *H. bacteriophora*. Fig. 1 shows the survival curves of cockroaches over 14 days after feeding the cockroaches on bait containing each nematode concentration. In the case of *P. americana*, the mortality rates of cockroaches fed with higher numbers of nematode-containing bait (20,000 and 100,000 nematodes per 0.3 g of bait) were significantly higher than that of the control ($P < 0.005$). The mortality rate of *P. fuliginosa* fed with only extremely high numbers of nematode-containing bait (100,000 nematodes per 0.3 g of bait) was significantly higher than that of the control ($P < 0.005$). No significant lethal effect on *P. japonica* was observed in any nematode-containing bait when compared with the control ($P > 0.05$). The mortality

rates of *B. germanica* at all tested concentrations were significantly higher than in the control ($P < 0.005$ or $P < 0.05$). Thus, the middle-stage instar of *B. germanica* was more susceptible to *H. bacteriophora* than that of *Periplaneta* species in our experimental design.

Because the insecticidal activity of *H. bacteriophora* largely depends on the associated bacterium *P. luminescens* (e.g., production of toxins), we performed a bacterial injection test to evaluate the pathogenicity of *P. luminescens* against *Periplaneta* species. Fig. 2 shows the survival curves of three *Periplaneta* cockroach species after injection of *P. luminescens* or *E. coli*. Injection of the nonpathogenic bacterium *E. coli* did not affect the survival of *Periplaneta* cockroaches. In contrast,

Fig. 1. Survival curves of the four cockroach species after being fed with bait containing several EPN concentrations. Survival curves are presented based on three to six experiments. (A) Lethal effect of *H. bacteriophora* on *P. fuliginosa*. *P. fuliginosa* was killed significantly only after being fed with a bait containing 100,000 EPNs when compared with control (0 EPN) (log-rank test, * $P < 0.05$, ** $P < 0.005$). (B) Lethal effect of *H. bacteriophora* on *P. americana*. *P. americana* was killed significantly after being fed with bait containing 20,000 and 100,000 EPNs when compared with control (0 EPN) (log-rank test, * $P < 0.05$, ** $P < 0.005$). (C) Lethal effect of *H. bacteriophora* on *P. japonica*. *P. japonica* was not killed significantly under any conditions when compared with control (0 EPN). (D) Lethal effect of *H. bacteriophora* on *B. germanica*. *B. germanica* was killed significantly at all tested concentrations (log-rank test, * $P < 0.05$, ** $P < 0.005$).

the EPN-mutualistic bacterium *P. luminescens* killed all cockroaches within 2 to 4 days (Fig. 2). Thus, *P. luminescens* is certainly pathogenic to *P. fuliginosa*, *P. americana*, and *P. japonica*, but *E. coli* is not.

Koehler *et al.* (1992) showed that the lethal effect of *S. carpocapsae* on cockroaches varied among species. In their experiments, *S. carpocapsae* killed *B. germanica* more effectively than *P. fuliginosa* and *P. americana*, which result is similar to the *H. bacteriophora* infection in our experiments. The cause of these different susceptibilities is unknown, but one possible reason is the different body sizes of *Periplaneta* and *B. germanica*. *Periplaneta* cockroaches are larger than *B. germanica*, and thus more EPNs might be required to kill them.

We found that injection of *P. luminescens* into the body cavity showed pathogenicity against *P. fuliginosa*, *P. japonica*, and *P. americana*. This suggests

that *Periplaneta* is susceptible to *P. luminescens*; however, *H. bacteriophora* was unable to effectively establish a lethal infection of *P. luminescens* in the bait experiment. Interestingly, Bowen and Ensign (1998) reported that oral administration of the protein complex extracted from *P. luminescens* was lethal to *B. germanica* but was not lethal to *P. americana*, while the injection of the protein complex was lethal to both species. This may suggest that the alimentary tract of *Periplaneta* cockroaches might be tolerant to toxins of *P. luminescens*. In our EPN infection test, although we need to study further which opening (anus, spiracles, or mouth of the host insect) the nematodes preferentially invade from, these different sensitivities to toxins may be another possible reason why *Periplaneta* cockroaches are resistant to *H. bacteriophora* infection.

We found that the injection of *P. luminescens* into the body cavity certainly killed *Periplaneta*

Fig. 2. Survival curves of the three *Periplaneta* cockroach species after injection with *E. coli* OP50 or *P. luminescens* TT01. Survival curves are presented based on three to five experiments. (A) Lethal effect of *P. luminescens* on *P. fuliginosa*. All cockroaches were killed within two days (log-rank test, $P < 0.005$). (B) Lethal effect of *P. luminescens* on *P. americana*. All cockroaches were killed within four days (log-rank test, $P < 0.005$). (C) Lethal effect of *P. luminescens* on *P. japonica*. All cockroaches were killed within two days (log-rank test, $P < 0.005$).

cockroaches, although even high concentrations of *H. bacteriophora* harboring *P. luminescens* did not effectively kill them in the nematode infection assay. Our results also indicate that the number of *H. bacteriophora* required to kill *B. germanica* is much less than that required to kill three *Periplaneta* cockroaches. A method to improve the delivery of *P. luminescens* into the insect body might be a key for successful biological control of cockroaches.

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