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Insecticidal activity of the metalloprotease AprA occurs through suppression of host cellular and humoral immunity

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ABSTRACT

The biochemical characterization of virulence factors from entomopathogenic bacteria is important to understand entomopathogen-insect molecular interactions. *Pseudomonas entomophila* is a typical entomopathogenic bacterium that harbors virulence factors against several insects. However, the molecular actions of these factors against host innate immune responses are not clearly elucidated. In this study, we observed that bean bugs (*Riptortus pedestris*) that were injected with *P. entomophila* were highly susceptible to this bacterium. To determine how *P. entomophila* counteracts the host innate immunity to survive within the insect, we purified a highly enriched protein with potential host insect-killing activity from the culture supernatant of *P. entomophila*. Then, a 45-kDa protein was purified to homogeneity and identified as AprA which is an alkaline zinc metalloprotease of the genus *Pseudomonas* by liquid chromatography mass spectrometry (LC-MS). Purified AprA showed a pronounced killing effect against host insects and suppressed both host cellular and humoral innate immunity. Furthermore, to show that AprA is an important insecticidal protein of *P. entomophila*, we used an *aprA*-deficient *P. entomophila* mutant strain (Δ*aprA*). When Δ*aprA* mutant cells were injected to host insects, this mutant exhibited extremely attenuated virulence. In addition, the cytotoxicity against host hemocytes and the antimicrobial peptide-degrading ability of the Δ*aprA* mutant were greatly decreased. These findings suggest that AprA functions as an important insecticidal protein of *P. entomophila* via suppression of host cellular and humoral innate immune responses.

1. Introduction

All organisms are continuously exposed to the threat of infection by environmental pathogens in the air, water, soil, and in food (Hejazi and Falkiner, 1997). These pathogens can cause serious diseases in host organisms, including humans (Villari et al., 2001). To defend host themselves, they have complex and well-developed immune systems (Accolla, 2006). However, environmental pathogens also have sophisticated strategies to counteract host immune systems (Ribet and Cossart, 2015; Vallet-Gely et al., 2008). One of these strategies is the secretion of highly toxic virulence factors. In particular, entomopathogenic bacteria are known to secrete insecticidal toxins to suppress insect immune systems and kill the host insect. There have been several reports on insecticidal toxins of entomopathogenic bacteria, and representative examples are Serralysin from *Serratia marcescens* and the Cry protein from *Bacillus thuringiensis* (Ishii et al., 2014b; Peterson et al., 2017). Both insecticidal toxins are fatal to host insects.

In general, multicellular animals have two types of immune systems, known as innate and acquired immunity, those are used to defend themselves against infectious organisms (Barton and Medzhitov, 2002; Medzhitov and Janeway, 1997). However, insects lack an acquired immune system, instead having a well-developed innate immune system that consists of both cellular and humoral innate immune responses. Cellular immunity is in insects is primarily mediated by hemocytes, which are macrophage-like cells that are involved in phagocytosis, nodulation

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and encapsulation (Schmidt et al., 2001; Strand and Pech, 2003). In contrast, humoral immunity is involved in the production of antimicrobial peptides (AMPs) and reactive oxygen species (ROS) to eliminate pathogens (Lemaitre et al., 1997; Lowenberger, 2001). This insect immunity has been primarily studied in holometabolous insects, such as *Drosophila melanogaster* and *Bombyx mori*, but a few studies have been done on hemimetabolous insects, such as *R. pedestris*.

The bean bug (*Riptortus pedestris*; Hemiptera: Alydidae) is a hemimetabolous insect that is a notorious soybean pest in Asia. This insect harbors a single gut symbiont of the betaproteobacterial genus *Burkholderia* in their specialized symbiotic organ, a posterior midgut fourth region named M4 (Kikuchi et al., 2005; Kim et al., 2013). *R. pedestris* has a well-developed immune system that has not been well-studied. Recently, our group purified and characterized three types of AMPs (riptocin, rip-thanatin, rip-defensin) from the hemolymph of bean bugs (Kim et al., 2015). In addition, two types of antimicrobial proteins were identified from the salivary gland (Rip-trialysin) and the midgut lysate (Cathepsin-L-like protease) of bean bugs (Byeon et al., 2015; Lee et al., 2017a). These antimicrobial substances produced by bean bugs effectively killed pathogens, such as *Escherichia coli* and *Staphylococcus aureus*. Although several immune-related substances have been identified in bean bugs (Kim et al., 2016), little is known about *Riptortus*-entomopathogen interactions. Thus, in this study, we investigated the interactions between the bean bug and *Pseudomonas entomophila* as a model entomopathogenic bacterium.

P. entomophila is a Gram-negative bacterium that was first isolated from the gut of flies in Guadeloupe (Vodovar et al., 2005). This bacterium has been shown to be highly virulent against *Drosophila* and other Lepidopteran insects (Liehl et al., 2006; Opota et al., 2011). The existence of a large set of genes in this bacterium that encode putative virulence factors, such as proteases, toxins, lipases, alginate synthase, and adhesion factors, was shown by whole genome sequence analyses (22). Among them, an insecticidal protein (AprA) was recently identified that protein belongs to the alkaline zinc metalloprotease family of the genus *Pseudomonas* (Liehl et al., 2006). This metalloprotease, which is secreted from *P. entomophila* cells, is known to regulated by the GacS/GacA two component system of the genus *Pseudomonas* (Liehl et al., 2006; Reimmann et al., 2005). There is one study about the local immune responses in the gut after oral infections and systemic infections of both wild-type and *aprA*-deficient (Δ*aprA*) mutant cells (Liehl et al., 2006). However, the molecular mechanisms of how AprA modulates host cellular and humoral innate immunity have not been examined.

In this study, we observed that *P. entomophila* is highly virulent to bean bugs, suggesting that this entomopathogenic bacterium secretes an unidentified virulence factor(s) upon systemic infection. Thus, we purified insecticidal protein from *P. entomophila* culture supernatant, and that protein was identified as AprA, which is metalloprotease. Finally, we addressed how this virulence factor modulates host innate immune responses *in vivo* and *in vitro*.

2. Materials and methods

2.1. Insect rearing

Bean bugs (*R. pedestris*) were reared in a room that was optimized for insect rearing, which was maintained at 28 °C under a long-day condition of 16 h light and 8 h dark as described previously (Jang et al., 2017). Briefly, bean bugs nymphs were reared in quadrangular plastic containers (34 cm × 19.5 cm wide and 27.5 cm high), supplemented with soybean seeds and distilled water (DWA) that contained 0.05% ascorbic acid. The plastic containers were cleaned every day, and fresh soybean seeds and DWA were replaced every two days. When adult bean bugs emerged from the nymphs, they were transferred to larger plastic containers (35 cm × 35 cm wide and 40 cm high) containing fresh soybean seeds and DWA. For egg laying by female bean bugs, several cotton pads were attached to the walls of the plastic rearing containers. Eggs laid on the cotton pads were collected every day and transferred to new small plastic containers for hatching.

2.2. Bacterial strains and culture media

The *Burkholderia* strain RPE75 is a spontaneous rifampicin-resistant mutant derived from *Burkholderia* strain RPE64 (Kikuchi et al., 2011). *Burkholderia* RPE75 cells were cultured in yeast-glucose (YG) medium (0.5% yeast extract, 0.4% glucose, and 0.1% NaCl) containing 30 μg/ml of rifampicin at 30 °C. *Escherichia coli* JM109 cells were cultured in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) at 37 °C. *Staphylococcus aureus* USA300 cells were cultured in tryptic soy broth (TSB) medium at 37 °C. *Pseudomonas entomophila* cells were cultured in Luria-Bertani (LB) medium containing 100 μg/ml of rifampicin at 30 °C. *P. entomophila ΔaprA* mutant cells were cultured in LB medium containing 100 μg/ml of rifampicin and 12.5 μg/ml of tetracycline (see Table 1).

2.3. Systemic infection and measurement of survival rates

The bacterial cells were cultured overnight in appropriate culture media. The overnight cultures were subcultured into fresh media and incubated for 4 h with vigorous shaking (180 rpm). The cultured bacterial cells were harvested by centrifugation at 6000 × g for 10 min and were washed with phosphate buffer to remove remaining antibiotics. Harvested cells were suspended in phosphate buffer, and 2 μl of bacterial solutions were used for systemic infections. Heat-killed bacterial cells were obtained by boiling the bacterial cells at 100 °C for 10 min. The *P. entomophila* culture supernatant was prepared by centrifugation of cultures at 6000 × g for 10 min followed by filtration of the supernatant through a 0.45-μm Ministart® syringe filter (Sartorius Stedim Biotech). Filtered culture supernatant was concentrated with a 10-kDa cutoff Vivaspin® concentrator (Sartorious Stedim Biotech), and the protein concentration in the culture supernatant was measured with a Bio-Rad Protein Assay solution (Bio-Rad). The culture supernatant proteins were used for systemic infections, as was purified AprA. Heat-treated culture supernatant and AprA were obtained by boiling samples at 100 °C for 5 min. Inactivated AprA was also obtained by treatment of the protein with 5 mM EDTA, after which the buffer was exchanged for Milli-Q water using a 30-kDa cutoff Amicon® concentrator (Merck Millipore). All prepared solutions were injected into the hemolymph of adult male bean bugs three days after they had molted into adults, and the survival rates were measured by counting dead insects.

2.4. Counting total number of hemocytes

Two microliters of phosphate buffer or bacterial suspension was injected into the hemolymph of adult male bean bugs, after which the hemolymph was extracted every 2 h. Extracted hemolymph was directly collected in an ice-cold tube containing decoagulation buffer (15 mM NaCl, 83 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 5% glycerol, pH 5.0) at a ratio of 1:9 (v/v, hemolymph to decoagulation buffer) to prevent melanization. Ten microliters of the hemolymph solutions were loaded onto a cytometer, and the number of hemocytes was counted under a microscope (Olympus BX50).