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Differential immunosuppression by inhibiting PLA_2 affects virulence of *Xenorhabdus hominickii* and *Photorhabdus temperata temperata*

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ABSTRACT

Immunity negatively influences bacterial pathogenicity. Eicosanoids mediate both cellular and humoral immune responses in insects. This study tested a hypothesis that differential bacterial virulence of *Xenorhabdus/Photorhabdus* is dependent on their inhibitory activity against phospholipase A_2 (PLA_2) activity. *P. temperata* subsp. *temperata* ('Ptt') was more than 40 times more potent than *X. hominickii* ('Xh'). Although both bacteria suppressed cellular immune responses, Ptt infection suppressed hemocyte nodule formation much more than Xh infection. Their differential immunosuppression appeared to be induced by their secondary metabolites because organic extracts of Ptt-cultured broth exhibited higher inhibitory activities against cellular immune responses than Xn-cultured broth extracts. Humoral immune responses were analyzed by measuring expression levels of 11 antimicrobial peptide (AMP) genes. Among inducible AMPs in hemocytes and fat body, higher number and more kinds of AMPs exhibited lower expression levels in Ptt infection than those in Xh infection. Suppressed immune responses induced by Ptt or Xh infection were significantly rescued by the addition of a catalytic product of PLA_2, suggesting that PLA_2 was a common inhibitory target. In fact, Ptt infection inhibited PLA_2 activity more strongly than Xh infection. RNA interference of a PLA_2 gene decreased its expression and significantly increased bacterial virulence. Moreover, addition of PLA_2 inhibitor to Xh infection enhanced its virulence, similar to virulence level of Ptt infection. These results suggest that variation in *Xenorhabdus/Photorhabdus* bacterial virulence can be explained by their differential inhibitory activities against host insect PLA_2.

1. Introduction

Like all other invertebrates, insects exhibit robust innate immunity to pathogens including virus, bacteria, parasitoid eggs, and fungi (Lemaitre and Hoffmann, 2007). Their integument serves as a mechanical barrier that is often considered as the first line of defense against infections (Zhukovskaya et al., 2013). Once passing through integument, pathogens confront with both cellular and humoral immune responses (Beckage, 2008). Circulating hemocytes in the hemocoel exert cellular responses that play a cardinal role in cleansing a major portion of pathogens from circulation shortly after infection. These cellular immune responses include phagocytosis, encapsulation, microaggregation, and nodulation (Lavine and Strand, 2002; Browne et al., 2013; Vlisidou and Wood, 2015). On the other hand, nonself recognition signal by pattern recognition molecules initiates humoral responses that comprise of induction of prophenoloxidase (PPO) cascade pathway and enhanced expression of antimicrobial peptide (AMP) genes (Marmaras and Lampropoulou, 2009). Based on *Drosophila* system, Toll and immune-deficiency (Imd) intracellular signaling pathways relay and amplify these recognition signals for the enhanced expression of AMP genes (Hultmark, 2003). These signals simultaneously activate serine protease enzymes for the activation of PPO that elicits intracellular signals through distinct membrane receptors (Jiang et al., 2009; An et al., 2009). Furthermore, recognition signals against pathogens are propagated to nearby hemocytes and fat body via immune mediators including eicosanoids (Gillespie et al., 1997).

Eicosanoids, a chemical group of oxygenated polyunsaturated C_{20} fatty acids deriving from arachidonic acid (AA), are essential mediators of insect innate immune responses (Kim et al., 2018). Catalytic reaction of phospholipase A_2 (PLA_2) on phospholipids (PLs) results in the production of arachidonic acid (AA) which is then converted into prostaglandins, epoxyeicosatrienoic acids, and leukotrienes by enzymatic activities of cyclooxygenase, epoxidase, and lipoxygenase, respectively (Burke and Dennis, 2009). In insects, these eicosanoids are involved in various physiological processes including secretion, reproduction, and immunity (Stanley and Kim, 2014). An experiment in *Manduca sexta* has shown that inhibition of this pathway using dexamethasone, a pharmaceutical PLA_2 inhibitor, renders test insects incapable of

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clearing pathogens from hemolymph while their immune response can be rescued with the addition of AA, suggesting that eicosanoids are actively involved in insect immunity (Stanley-Samuelson et al., 1991). Interestingly, some intruders have evolved to suppress their immune responses by targeting PLA_2, a pivotal enzyme involved in the very first step of the generation of eicosanoids (Kim et al., 2005).

Xenorhabdus hominickii (Xh) and *Photorhabdus temperata temperata* (Ptt) are Gram-negative insect pathogens that are mutualistic to entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis megidis*, respectively (Akhurst, 1980; Boemare, 2002; Kang et al., 2004). After entering target insect's hemocoel through natural openings including spiracle, mouth, and anus, infective juveniles will release symbiont bacteria from their intestine into host hemocoel. These bacteria eventually will suppress host immune system by causing hemolysis, restricting hemocyte-spreading and nodulation, inhibiting PPO activation, degrading AMPs, and suppressing eicosanoid biosynthesis (Park and Kim, 2000; Shrestha and Kim, 2008; Sadekuzzaman et al., 2017a). Under such immunosuppressive situation, bacterial proliferation and toxin production will result in septicemia and kill host insects within 24 h post infection (Poinar, 1979; Akhurst and Dunphy, 1993). These entomopathogenic bacteria specifically target PLA_2 enzyme and inhibit its activity which will result in shutdown of eicosanoid biosynthesis (Kim et al., 2005; Park et al., 2017). Xh can inhibit insect immune responses by reducing hemocyte nodulation, phenoloxidase, and PLA_2 enzyme activities that can be rescued by addition of AA (Sadekuzzaman et al., 2017a). These bacteria can also suppress expression levels of AMP genes such as attacin-1, attacin-2, defensin, gallerimycin, and transferrin-1 of *Spodoptera exigua*, one of its target host insects (Sadekuzzaman et al., 2017a). Furthermore, oxindole, a secondary metabolite of these bacteria, can suppress hemocyte-spreading behavior (Sadekuzzaman et al., 2017a). Ptt also contains immunosuppressive compounds that can inhibit hemocyte behavior and nodulation (Seo and Kim, 2009). In all cases, immune responses can be rescued by the addition of AA and other eicosanoids. These results indicate that immunosuppression of these bacteria is induced by inhibition of PLA_2 enzyme activity of the target insect.

There is a variation in bacterial virulence between *Xenorhabdus* and *Photorhabdus* (Givaudan and Lanois, 2016). Although immunosuppression is known to be required for bacterial pathogenicity, it is currently unclear how much immunosuppressive activity of these bacteria contributes to their virulence. This study demonstrated that differential immunosuppression between Xh infection and Ptt infection was due to their difference in inhibiting activities of PLA_2. It then tested a hypothesis that differential virulence between Xh and Ptt was due to their difference in PLA_2 inhibition. Finally, the causal relationship between bacterial virulence and PLA_2 inhibitory activity was validated by modulating bacterial virulence after adding eicosanoid biosynthesis substrate or inhibitor.

2. Materials and methods

2.1. Bacterial culture and insect rearing

Xh and Ptt were isolated from *S. monticolum* and *H. megidis*, respectively. They were grown in tryptic soy broth (TSB: Difco, Sparks, MD, USA) at 28 °C for 48 h (Kang et al., 2004; Park et al., 2017). Bacterial dose for pathogenicity test was evaluated as colony-forming unit (CFU) by spreading fresh-cultured broth on Luria-Bertani (LB) agar medium (Difco) and subsequently culturing at 28 °C for 48 h. *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) was cultured in LB medium overnight at 37 °C with shaking at 200 rpm. Bacteria were killed with heat treatment (80 °C for 10 min) and their cell numbers were counted using a hemocytometer under a phase contrast microscope (BX41, Olympus, Tokyo, Japan). Bacterial suspension was serially diluted with sterilized and deionized distilled H_2O to prepare different concentrations of bacteria.

A laboratory colony of *S. exigua* was used in this study. They were reared at 25 ± 1 °C with 16 h light: 8 h dark condition. Larvae were fed an artificial diet (Goh et al., 1990) while adults were fed on a 10% sucrose solution. All experiments used fourth (L4) and fifth (L5) instar larvae.

2.2. Chemicals

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), prostaglandin E_2 (PGE_2: 5Z,11α,13E,15S)-11,15-dihydroxy-9-oxoprosta-5,13-dienoic acid), and benzylideneacetone (BZA: (E)-4-phenyl-3-buten-2-one acetic acid) were purchased from Sigma-Aldrich Korea (Seoul, Korea) and dissolved in dimethyl sulfoxide (DMSO). A PLA_2 surrogate substrate, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycerol-3-phosphatidylcholine, was purchased from Molecular Probes, Inc. (Eugene, OR, USA) and dissolved in high grade ethanol (Sigma-Aldrich Korea). Anticoagulant buffer (ACB, pH 4.5) was prepared with 186 mM NaCl, 17 mM Na_2EDTA, and 41 mM citric acid.

2.3. Organic extracts of bacterial cultured broth

Xh or Ptt bacteria were cultured in TSB at 28 °C for 48 h. Culture broths were centrifuged at 12,500*g* for 30 min and supernatants were used for subsequent fractionation. At the first step, the same volume (1 L) of hexane was mixed with the supernatant and separated into organic and aqueous fractions. Organic hexane extract ('HEX') was combined and dried with a rotary evaporator (Sunil Eyela, Seongnam, Korea) at 40 °C. The resulting 0.02 mg was obtained from 1 L cultured broth and resuspended with 5 mL of methanol. The aqueous phase was then combined with 1 L of ethyl acetate. Ethyl acetate extract ('EAX') was also dried with the rotary evaporator at 40 °C and the resulting extract (0.2 mg) was resuspended with 5 mL of methanol. The final aqueous fraction represented aqueous extract ('AQU').

2.4. Bacterial virulence assays against S. exigua larvae

For dose-mortality analysis, seven different concentrations ($10–10^7$ CFU/larva) of Xh and Ptt bacteria were prepared from freshly cultured bacteria by diluting with sterilized 50 mM phosphate-buffered saline (PBS, pH 7.4). After surface sterilization with 95% ethanol, each L5 *S. exigua* was injected with 3 μL of bacterial suspension into the first abdominal proleg using a microsyringe (Hamilton, Reno, Nevada, USA) and incubated at 25 °C. Mortality was observed at 30 h post-infection (PI). For time-mortality analysis, 10^5 colony-forming unit (CFU) bacteria of Xh or Ptt were injected to each L5 larva of *S. exigua* and incubated at 25 °C. Mortality was measured every 24 h PI. For pharmacological test, 0–100 μg/μL of AA or BZA were prepared in DMSO. Different concentrations of AA or BZA were injected into L5 larvae along with Xh or Ptt to monitor changes in mortality. Each treatment consisted of three replicates and each replication used 10 larvae.

2.5. Assessment of nodule formation

Nodule formation assay was performed by injecting live or heat-killed bacteria. Heat-killing was confirmed by plating treated bacteria on LB plate and culturing at 28 °C for 48 h. At 8 h PI of 10^5 bacterial cells, nodules were counted by dissecting the larvae under a stereomicroscope (Stemi SV11, Zeiss, Jena, Germany) at 50 × magnification. Each treatment used 10 larvae.

2.6. Measurement of PLA_2 enzymatic activity

For measuring PLA_2 activity, L5 larvae of *S. exigua* were injected with Xh or Ptt bacteria and incubated at 25 °C. At 8 h PI, hemolymph was collected from 10 larvae per experimental unit. Hemocytes and plasma were separated by centrifugation at 800*g* for 3 min at 4 °C.