Sequential Immunosuppressive Activities of Bacterial Secondary Metabolites from the Entomopathogenic Bacterium *Xenorhabdus nematophila*

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The entomopathogenic bacterium *Xenorhabdus nematophila* secretes at least eight bacterial metabolites that play crucial roles suppressing target insect immune responses by inhibiting eicosanoid biosynthesis. We analyzed sequential changes in bacterial metabolite production during bacterial growth and analyzed their individual immunosuppressive activities against the insect host, *Spodoptera exigua*. *X. nematophila* exhibited a typical bacterial growth pattern in both insect host and culture medium, and eight metabolites were secreted at different time points. At the early growth phase (6–12 h), Ac-FGV and PHPP were detected in significant amounts in the culture broth. At this early phase, both Ac-FGV (18 μg/ml) and oxindole (110 μg/ml) levels significantly inhibited hemolymph. At the late growth phase (12–36 h), all eight metabolites were detected at significant levels (10–140 μg/ml) in the culture broth and were sufficient to induce hemocyte toxicity. These results suggest that *X. nematophila* sequentially produces immunosuppressive metabolites that might sequentially and cooperatively inhibit different steps of insect immune responses.

**Keywords:** insect immune, immunosuppression, *Xenorhabdus nematophila*, hemocyte, benzylideneacetone

**Introduction**

Insect immunity is innate and highly efficient at defending against microbial pathogens such as bacteria, viruses, and fungi (Lavine and Strand, 2002). Upon pathogen infection, insects recognize the invading pathogen based on the surface molecular pattern and express pathogen-specific immune responses with the help of immune mediators, such as cytokines and eicosanoids (Gillespie et al., 1997). Immune responses are usually initiated by cellular responses and subsequently executed by antimicrobial humoral responses. Phagocytosis and nodulation are the main cellular immune responses against bacterial infection (Horohov and Dunn, 1983; Nappi and Vass, 1998). Peptidoglycan bacterial cell wall components specifically induce the expression of antimicrobial peptides (AMPs), which mainly perform the insect humoral immune responses (Hultmark, 2003; Lemaitre and Hoffmann, 2007).

The entomopathogenic bacterium *Xenorhabdus nematophila* is Gram-negative and mutualistic to the nematode *Steinernema carpocapsae* (Steinernematidae) (Akhurst, 1980; Park et al., 1999). Host nematodes enter the target insect hemocoel through natural openings such as the mouth, anus, and trachea (Kaya and Gaugler, 1993). When the infective juveniles enter target insects, they release symbiotic bacteria into the hemocoel, where the bacteria inhibit insect immune responses and then multiply (Park and Kim, 2000; Ji and Kim, 2004). Subsequent bacterial food signals induce nematode reproduction and proliferation (Park and Forst, 2005). Thus, insect immunosuppression is the main mutualistic tool for a successful nematode–bacterial complex life cycle. Various immunosuppressive actions against cellular and humoral immune responses have been reported for *X. nematophila*. First, *X. nematophila* immunosuppresses its host by inhibiting AMP gene expression. The main antimicrobial peptide against *X. nematophila* is cecropin (Aymeric et al., 2010), which is inhibited in its expression by *X. nematophila* infection (Ji and Kim, 2004). Second, immunosuppression by *X. nematophila* is executed by directly killing hemocytes. *X. nematophila* inhibits cellular immunity by being toxic to hemocytes (Ribeiro et al., 2003). Indeed, *X. nematophila* produces a cytotoxin that triggers hemocyte apoptosis (Cho and Kim, 2004; Vigneux et al., 2007). Third, immunosuppression by *X. nematophila* is accomplished by inhibiting eicosanoid biosynthesis. Eicosanoids are a group of C20 oxygenated fatty acids derived from arachidonic acid that play diverse roles mediating immune signals (Stanley and Kim, 2011). *X. nematophila* inhibits the catalytic activity of phospholipase A₂ (PLA₂), which releases arachidonic acid from a phospholipid substrate (Park and Kim, 2000; Shrestha et al., 2010). Five novel immunosuppressive bacterial metabolites (oxindole, indole, p-hydroxypropionic acid [PHPP], cyclo-Pro-Tyr [cPY], 4-hydroxyphenylacetic acid [HPA]) of *X. nematophila* have been identified and significantly inhibit hemocyte PLA₂ activity (Seo and Kim, 2011). Considering the previously known three PLA₂ inhibitors of benzylideneacetone (BZA), Pro-Tyr (PY), and acetylated Phe-Gly-Val (Ac-FGV) from *X. nematophila* (Shrestha et al., 2010), *X. nematophila* produces and secretes at least eight immunosupp-

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Bacterial metabolites were synthesized from 48 h culture broth, are synthesized during different bacterial growth phases in target insect hemocoel and provide differential immunosuppressive activity. However, little is known about their synthesis and release during bacterial growth. Furthermore, no comparative biological activity analysis has been performed among the eight bacterial metabolites.

We determined the sequential biosynthesis and release of the eight bacterial metabolites during bacterial growth of *X. nematophila*. We also analyzed their differential immunosuppressive activities and various immune-associated responses. Ultimately, we explain the sequential and cooperative action of the eight *X. nematophila* bacterial metabolites to effectively induce host immunosuppression against insect immune responses.

**Materials and Methods**

**Bacterial culture and insect rearing**

*X. nematophila* K1 was isolated from the entomopathogenic nematode *S. carpocapsae* (Park et al., 1999) and cultured on tryptic soy broth (TSB; Difco, USA) at 28°C for 48 h. The bacterial culture was centrifuged at 4,000 rpm for 10 min and washed with sterilized 50 mM phosphate buffer saline (PBS, pH 7.4). *S. exigua* were reared in the laboratory under 25±1°C and 16 h light: 8 h dark conditions. *S. exigua* larvae were fed an artificial diet (Goh et al., 2012). Eight bacterial metabolites were extracted from 1 L of bacteria-culture broth using the method of Seo et al. (2012). Samples were cleaned with a PTFE syringe filter (Cronus, UK). Ten microliter of cleaned sample was injected into an HPLC equipped with a C18 column (Deltapak, 15 mm, 300 A, 300×7.8 mm). The samples were then separated in a mobile phase of methanol: water (60:40, v/v) at a flow rate of 0.5 ml/min for 30 min using a UV detector (Waters) at 254 nm.

**Bacterial growth curve analysis**

Two microliters (100 CFU) of freshly cultured *X. nematophila* was injected by microsyringe (Hamilton, USA) through the hemocoel into each fifth instar larva. Considering a 200 μl larval hemocoel volume in *S. exigua* (Kim and Kim, 2010), 5×10^5 CFU of bacteria were inoculated into 1 L TSB broth. After a 6, 12, 18, 24, 36, and 48 h incubation, culture medium and hemolymph from the larvae were spread on a tryptic soy agar plate and the number of colonies was counted by the standard plate culture method.

**Bioactive metabolites from *X. nematophila* culture broth**

Bacterial metabolites were extracted from 1 L of bacteria-culture broth using the method of Seo et al. (2012). Eight bacterial metabolites include BZA, proline-tyrosine (PY), Ac-FGV, 1H-benzo[b]pyrrole (indole), 2-indolinone (oxindole), PHPP, HPA, and cPY were extracted. All samples were dissolved in dimethyl sulfoxide (DMSO) (BZA, PY, FGV, HPA, indole, and oxindole) or methanol (PHPP and cPY) for the bioactivity assay and prepared at different concentrations (0.1, 1, 10, 100, 1,000, and 10,000 μg/ml).

**Reverse-phase high performance liquid chromatography (HPLC) analysis**

Both hexane and ethyl acetate extracts of the *X. nematophila* culture broth were analyzed by HPLC (Waters, USA). Samples were cleaned with a PTFE syringe filter (Cronus, UK). Ten microliter of cleaned sample was injected into an HPLC equipped with a C18 column (Deltapak, 15 mm, 300 A, 300×7.8 mm). The samples were then separated in a mobile phase of methanol: water (60:40, v/v) at a flow rate of 0.5 ml/min for 30 min using a UV detector (Waters) at 254 nm.

**Nodule formation assay**

The nodule formation assay was performed by injecting 2 μl of 10^4 *Escherichia coli* Top10 cells (Invitrogen, USA), through the abdominal proleg into the *S. exigua* hemocoel using a microsyringe as described previously (Park and Kim, 2000). *E. coli* was chosen because it is not pathogenic to insects, presumably due to the lack of production of PLA2-inhibitory metabolites. After an 8 h incubation at 25°C, test insects were dissected, and the number of melanized nodules was counted under a stereo microscope (SZX9, Olympus, Japan). A 2 μl aliquot of different concentrations of each sample was injected into each larva along with *E. coli*, and the resulting nodules were counted as described above. Each treatment was independently replicated five times.

**Measurement of phenoloxidase (PO) activity**

Hemolymph PO activity was determined using L-3,4-dihydroxyphenylalanine (DOPA) as the substrate (Kim et al., 2001). Hemolymph was collected into 1.5 ml microtubes by cutting the abdominal proleg. The sample solution consisted of 1 μl of test material and 9 μl of hemolymph and was incubated for 10 min at 25°C. The PO substrate solution was prepared in 990 μl of PBS containing 20 μg/μl DOPA in acetone. The PO reaction was initiated by adding the PO substrate solution to the sample solution. The initial absorbance change was monitored at 495 nm using a spectrophotometer.

**Measurement of PLA2 activity**

Hemocyte PLA2 activity was fluorometrically determined with a pyrene-labeled phospholipid substrate [1-hexadecanoyl-2-(1-pyrenecanoyl)-sn-glycerol-3-phosphatidylcholine] in the presence of bovine serum albumin (BSA), as described by Radvanvi et al. (1989). The fluorescent phospholipid was prepared at a concentration of 0.2 mM in ethanol. Hemolymph was collected into 1.5 ml microtubes containing a few granules of phenylthiourea and centrifuged at 4,000 rpm for 10 min. The plasma was removed and washed three times with washing buffer (50 mM Tris-HCl; pH 7.0, 100 mM NaCl, and 1 mM EDTA). The hemocyte pellet was resuspended in the washing buffer and homogenized by three ultrasonicators (Bandelin Sonoplus, Germany) cycles for 10 min at 75% power. The protein concentration in the hemocyte extracts was measured by the Bradford method (Bradford, 1972) using BSA as the standard. The reaction mixture was prepared in a 96 well microplate by adding 142.5 μl Tris buffer, 1 μl 1 M CaCl₂, 1 μl 10% BSA, and 2 μl 0.2 mM substrate. The sample solution consisted of 1 μl test bacterial metabolites and 1 μl hemocyte enzyme extract (200 μg protein), which was incubated for 20 min at room temperature. The PLA2 reaction was initiated by adding the sample sol-