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Sequential Immunosuppressive Activities of Bacterial Secondary Metabolites from the Entomopahogenic 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 phenoloxidase and phospholipase A_2 activities in *S. exigua* 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 A_2 (PLA_2), 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 PLA_2 activity (Seo and Kim, 2011). Considering the previously known three PLA_2 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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ressive metabolites. All of these metabolites, which can be identified 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*., 1990), and adults were fed a 10% sucrose solution. Fifth instar larvae were used for the bioassay and hemolymph collection.

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 nodulation 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 PLA_2-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 PLA_2 activity

Hemocyte PLA_2 activity was fluorometrically determined with a pyrene-labeled phospholipid substrate [1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycerol-3-phosphatidylcholine] in the presence of bovine serum albumin (BSA), as described by Radvanyi *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 ultrasonicator (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 $CaCl_2$, 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 PLA_2 reaction was initiated by adding the sample sol-

ution to the reaction mixture. Fluorescence intensity was monitored with an Aminco Bowmen Series 2 luminescence spectrometer (FA257, Spectronic Instruments, USA) using excitation and emission wavelengths of 345 and 394 nm, respectively. Enzyme activity was calculated in pmol/min using a formula according to Ravanyi *et al*. (1989).

Cytotoxicity measurement

The cytotoxic effect of each bioactive metabolite was evaluated by the MTT (3-14, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which was performed as described previously (Boonsuepsakul *et al*., 2008). The Sf9 insect cell line was used as the test target cell because it has been used for other insecticidal toxin analyses (Fraga *et al*., 2005). Sf9 cells were cultured in TC-100 insect cell culture medium (Welgene, Korea) containing 10% fetal bovine serum. Each sample (2.5 µl) was added to the cells at the subconfluent growth phase and incubated at 30°C. After a 24 h incubation, 30 µl of MTT (3 mg/ml) was added to each well, and the cells were cultured for another 3 h. The formazan granules that formed in viable cells were dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in a microplate reader.

Data analysis

All assay data were square root and arcsine transformed for normalization. Treatment means and variances of the transformed data were analyzed by PROC GLM in the SAS program (SAS Institute, 1989). Means were compared with the least significant difference test at a Type I error of 0.05. The medium inhibition concentrations (I_{50}s) of individual experiments were calculated by probit analysis using the EPA Probit Analysis Program, ver. 1.5 (U.S. Environmental Protection Agency, USA).

Results

Synthesis and release of the eight bacterial metabolites into plasma of the target insect were measured at different times after infection to determine individual effects on host physiology. However, it was difficult to detect and quantify these metabolites in the infected insect host due to their small volume (~200 µl in the *S. exigua* instar). Instead, we cultured the bacteria in normal bacterial culture medium and measured the metabolite concentrations by HPLC after extracting the solvent.

Comparison of *X. nematophila* growth patterns in the insect host and culture medium

X. nematophila grew in both the insect hosts and TSB. Bacteria in both cultures reached a maximum of 8.2×10^8 CFU/ml (Fig. 1). *X. nematophila* grew slowly in the insects during the first 12 h post-infection (PI). The population increased exponentially until 18 h PI and then began to die. In TSB, *X. nematophila* showed exponential growth at 6 h after inoculation, which lasted for 36 h. Thus, bacterial growth during the first 6 h in TSB was matched to that of the first 12 h in the insect host. Based on this comparison between bacterial growth patterns in insects and TSB, we divided bacterial growth into two phases of an early lag phase ("0–12 h" in insects, "0–6 h" in TSB) and a late exponential phase ("12–18 h" in insects, "6–36 h" in TSB). Hemocyte density decreased during the late phase. The hemocyte population decreased significantly in the infected insects after 12 h PI (Fig. 1), suggesting that the late exponential growth phase might be when synthesis and accumulation of immunosuppressive metabolites was most active. Thus, the metabolite analysis in TSB cultures was divided into two subcategories of an early exponential phase (6–12 h) and a late exponential phase (12–36 h).

Bacterial metabolite production during *X. nematophila* growth

Temporal synthesis and release of the eight bacterial metabolites were analyzed in the *X. nematophila* TSB culture broth (Fig. 2). The eight compounds were divided into hexane and ethyl acetate fractions based on their polarity (Fig. 2A). No bacterial metabolites were detected at significant levels in the bacterial culture broth until the first 6 h of culture (Fig. 2B). However, Ac-FGV and oxindole were detected at significant levels of 18 µg/ml and 110 µg/ml, respectively, at 12 h. During the late exponential growth phase (12–36 h), all metabolites accumulated in the culture media. By 48 h, all metabolites showed their maximum concentrations. PHPP was the most abundant metabolite, and BZA was the least abundant (Fig. 3B).

Variations in the immunosuppressive activities of the eight bacterial metabolites

The eight bacterial metabolites were analyzed for their inhibitory activity against immune-associated processes (Fig. 3A). All eight bacterial metabolites significantly inhibited hemocyte nodulation, and BZA was the most potent (Fig. 3B). Typical nodulation was well illustrated in a previous

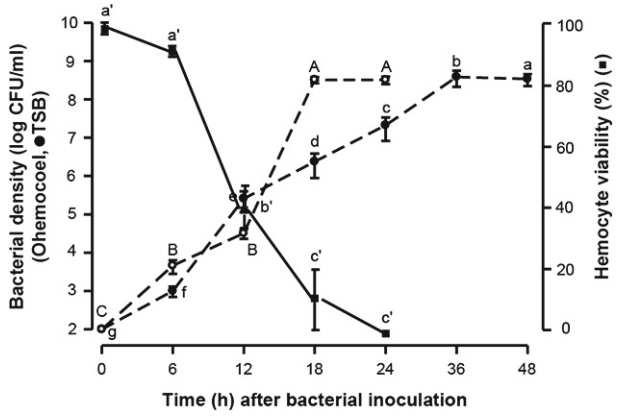

Fig. 1. Bacterial growth and toxicity to hemocytes of fifth instar *S. exigua*. The primary form of *X. nematophila* was inoculated into the fifth instar at 1×10^2 CFU/larva or to TSB bacterial culture medium at 5×10^5 CFU/ml. Hemolymph or culture broth was collected at different times after inoculation and used to count the bacteria and hemocytes (for injection into insects). Each time point measurement was independently replicated three times. Different letters for each treatment type represent significant difference among means at a Type I error =0.05 (LSD test).

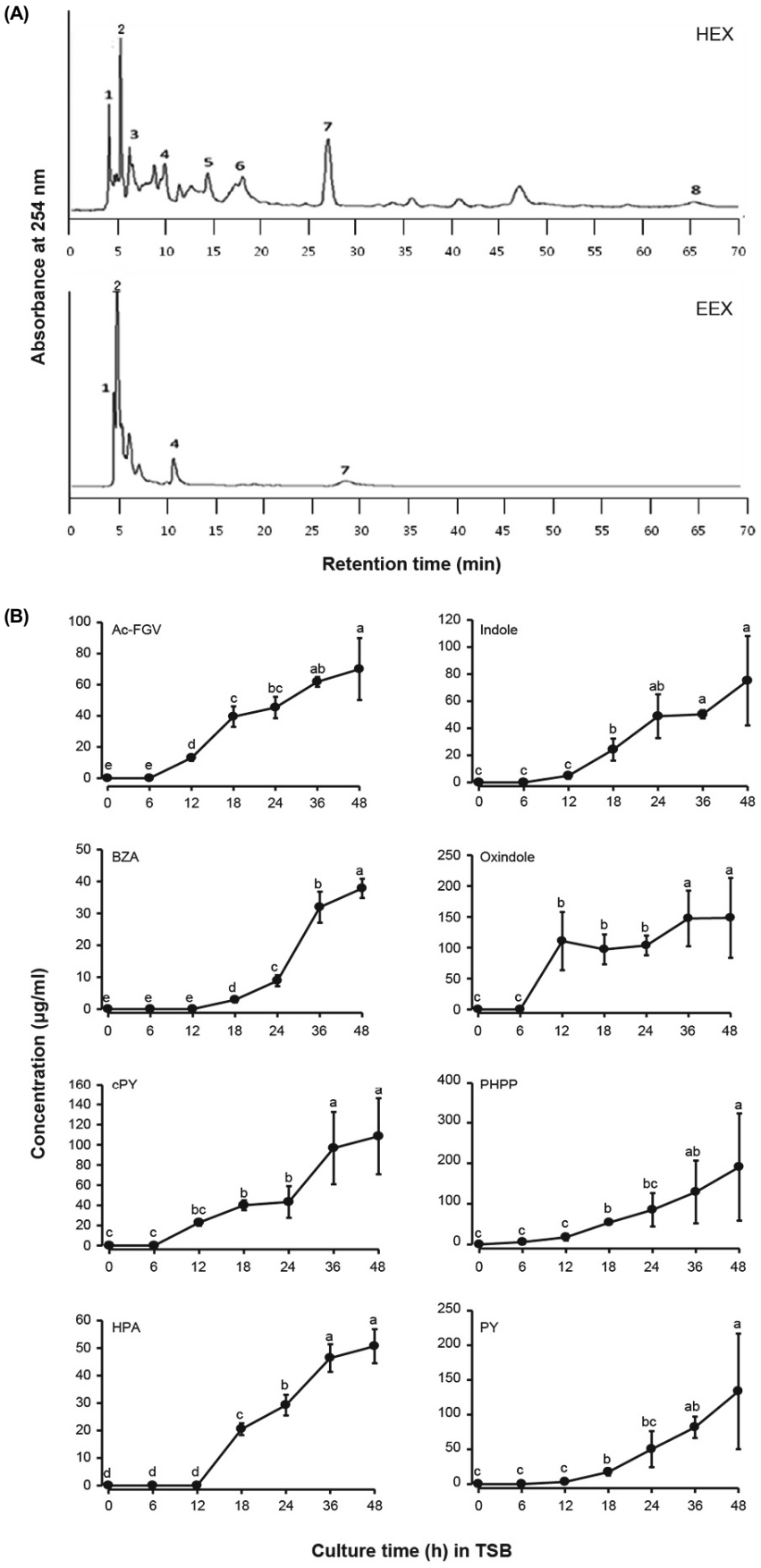

Fig. 2. Secondary metabolites in *X. nematophila* culture broth. The bacteria (5×10^3 CFU) were inoculated into 1 L of TSB culture media and cultured at 28°C. Each time measurement used three independent culture broth values. (A) Chromatograms of the hexane extract ('HEX') and ethyl acetate extract ('EEX'). The known peaks are PHPP (1), Ac-FGV (2), PY (3), cPY (4), HPA (5), indole (6), oxindole (7), and BZA (8). (B) Concentrations of the eight metabolites. Each concentration was the sum of the concentrations of the two extracts. Different letters above the means at a Type I error=0.05 (LSD test) indicate significance.

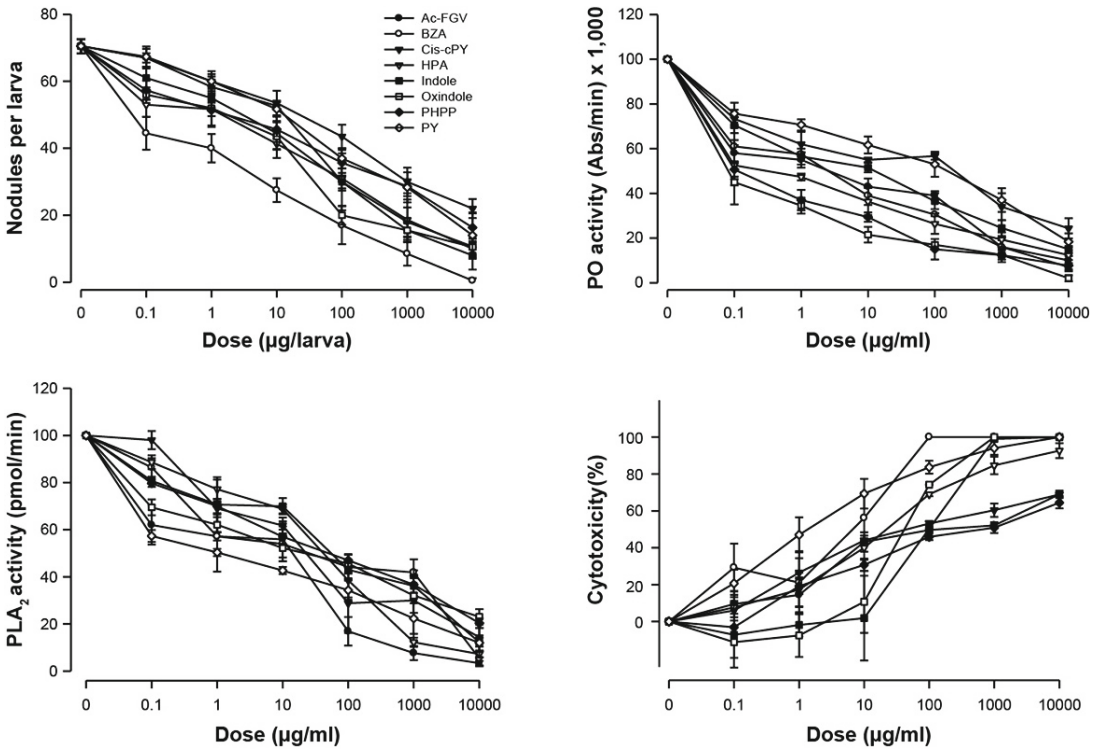

Fig. 3. Inhibitory activities of eight bacterial metabolites against immune-associated responses of *S. exigua*. (A) Nodulation assay. Different concentrations of bacterial metabolites were injected into *S. exigua* larvae along with *E. coli* (2×10^4 cells). Nodules were counted in the hemocoel after 8 h at 25°C. Control (0 μg) represents solvent injection with *E. coli*. Each measurement used five larvae. (B) Phenoloxidase (PO) activity assay. Hemolymph (9 μl) was incubated with 1 μl of different concentrations of test metabolites. The remaining PO activity was assessed by adding substrate solution containing DOPA. Each treatment was replicated three times. (C) PLA_2 enzyme activity assay. The PLA_2 enzyme was extracted from hemocytes of fifth instar *S. exigua* larvae. The enzyme extract (1 μl contained 50 μg protein) was mixed with 1 μl of different concentrations of the bacterial metabolites and incubated for 20 min at 25°C. Remaining enzyme activity was measured by adding the substrate solution containing pyrene-labeled phospholipid. Each treatment was replicated three times. (D) Cytotoxicity assay. Sf9 cells were placed in 96 well plate and treated with different concentrations of bacterial metabolites. MTT was added to the cells after 24 h at 30°C. The plates were read at 570 nm after another 3 h.

study (Stanley and Km, 2011). Efficient nodulation of hemocytes requires PO activity (Cerenius and Söderhäll, 2004). All eight metabolites significantly inhibited PO activity, and oxindole and PHPP were highly potent. PO activity in *S. exigua* is induced by eicosanoids, which are synthesized by PLA_2 (Shrestha and Kim, 2009). The eight bacterial metabo-

Table 1. Effects of the eight *X. nematophila* bacterial metabolites (BM) on immune-associated responses of *S. exigua*. Median inhibitory concentration (I_{50}) was estimated.

BM	BM (μg/ml) at 48 h TSB	I_{50} (95% CI)[a]			
		Nodulation (μg/larva)	Phenoloxidase (μg/ml)	PLA_2 (μg/ml)	Cytotoxicity (μg/ml)
Ac-FGV	70.0 ± 19.9	101.33[b] (46.59 – 239.37)	1.64[c] (0.52 – 4.01)	25.37[ab] (15.73 – 40.70)	255.45[a] (43.56 – 3785.15)
BZA	56.8 ± 3.0	1.47[c] (0.68 – 2.80)	1.59[bc] (0.60 – 3.48)	1.89[b] (0.05 – 13.82)	2.25[c] (0.04 – 28.73)
cPY	108.4 ± 37.7	105.69[b] (63.03 – 182.35)	38.58[a] (14.45 – 105.43)	45.28[ab] (9.56 – 236.07)	96.49[a] (76.55 – 121.64)
HPA	44.9 ± 12.3	23.82[b] (11.89 – 47.06)	0.31[c] (0.05 – 1.08)	45.69[a] (21.06 – 101.64)	24.23[bc] (15.36 – 38.09)
Indole	75.1 ± 33.0	489.72[a] (252.89 – 1064.91)	6.13[b] (2.42 – 13.68)	35.12[ab] (4.88 – 261.98)	128.10[ab] (22.60 – 347.58)
Oxindole	148.3 ± 64.7	29.24[b] (16.95 – 50.29)	0.05[c] (0.01 – 0.17)	38.34[ab] (11.75 – 97.52)	521.81[a] (85. 52 – 1671.35)
PHPP	190.8 ± 132.3	42.78[b] (23.02 – 80.41)	0.09[c] (0.02 – 0.32)	15.60[ab] (5.08 – 43.60)	44.63[b] (35.02 – 56.87)
PY	133.6 ± 83.2	195.08[ab] (112.08 – 357.83)	55.31[a] (25.03 – 127.38)	1.07[b] (0.25 – 3.06)	1.75[c] (1.02 – 2.84)

[a] Comparison was based on the non-overlapping 95% confidence interval (CI). Different letters indicate a significant difference among bacterial metabolites.

lites inhibited PLA_2, and BZA and PY were the most potent. Finally, the cytotoxicity of these bacterial metabolites was assessed in Sf9 cells. BZA and PY exhibited the most potent cytotoxicity.

Discussion

The synergistic interaction between nematodes and bacteria is well demonstrated by the *S. carpocapsae* and *X. nematophila* system. The crucial synergistic activity of *X. nematophila* is suppression of the target insect immune response to protect the host nematode as well as the bacteria themselves (Dunphy and Webster, 1984). Insects defend against bacterial infection using both cellular and humoral immune responses (Gillespie *et al*., 1997). Phagocytosis and nodulation are effective for inhibiting bacterial growth (Ratcliffe and Gots, 1990). Eicosanoids mediate these cellular immune responses (Shrestha and Kim, 2008; Stanley and Kim, 2011), and their biosynthesis is inhibited by *X. nematophila* bacterial metabolites (Kim *et al*., 2005). Our previous study (Seo *et al*., 2012) identified four novel PLA_2 inhibitors in 48 h bacterial culture broth of *X. nematophila*. Our present study quantified eight bacterial metabolites from *X. nematophila* during different culture periods and analyzed their immunosuppressive activities against *S. exigua*.

Bacterial growth of *X. nematophila* was compared in insects and TSB medium to extrapolate the bacterial metabolites synthesized in TSB medium to the infected insects. The initial slow growth phase of *X. nematophila* was shorter in TSB (0–6 h) than that in insects (0–12 h), suggesting that there may be a significant immune defense response by the infected *S. exigua* to suppress bacterial growth. Few hemocytes underwent cell death during this period, but hemocytes suffered significant death after 12 h due the cytotoxic *X. nematophila* compounds. To explain these observations, the eight bacterial metabolites were quantified in bacterial culture broth cultured for different time periods. The early 6 h culture broth did not contain significant amounts of bacterial metabolites. PHPP and Ac-FGV were detected in culture broth at 12 h. It was notable that PHPP was a potent inhibitor of PO and PLA_2 at low concentrations. PHPP had median inhibitory concentration (I_{50}) values of 0.09 μg/ml against PO and 15.6 μg/ml against PLA_2. PHPP was detected at 110 μg/ml during the early phase. Thus, PHPP inhibited both enzyme activities. Similarly, Ac-FGV had I_{50} values of 1.64 μg/ml against PO and 25.37 μg/ml against PLA_2. Ac-FGV was detected at 18 μg/ml during the early phase, and effectively inhibited PO. *S. exigua* exhibits phagocytosis and nodulation upon bacterial challenge (Park and Kim, 2000; Shrestha and Kim, 2007). Small G proteins are involved in cellular immunity and are associated with eicosanoids (Stanley and Kim, 2011). Small GTP-binding proteins have been postulated to mediate the link between the nonself recognition signal triggered by hemocyte surface receptors or extracellular pattern recognition molecules and intracellular signaling pathways in insect hemocytes (Marmaras and Lampropoulou, 2009). The small G proteins that most clearly function in signal transduction include Ras, Ras-related proteins (Ral and Rap), and Rho/Rac/Cdc42 proteins, which are annotated in *Drosophila*, although their immunological functions are little understood in insects (Zettervall *et al*., 2004). Two members of the Rho GTPase family, Rac1 and Rac2, regulate cytoskeletal rearrangement and migration of *Drosophila* hemocytes (Paladi and Tepass, 2004; Williams *et al*., 2007). Ras/mitogen-activating protein kinase signaling appears to be a downstream signal involved in hemocyte phagocytosis (Foukas *et al*., 1998; Winter *et al*., 2007). Hemocyte phagocytosis requires a cytoskeletal rearrangement to exhibit the characteristic cytoplasmic extensions (Gupta and Campenot, 1996). The small G protein Rac1 in *S. exigua* has been implicated in cytoskeletal rearrangement of hemocytes to increase its mobility in the hemocoel in response to pathogen infection (Kim and Kim, 2010). In addition, Rac1 activity is necessary for phagocytosis, which is under the control of eicosanoids (Kim *et al*., 2009). In addition, *S. exigua* Ras is implicated in cellular immune responses and PO activity, in which its immune-mediating activity is dependent on eicosanoids (Lee *et al*., 2011). PO activity is required for hemocyte nodule formation by catalyzing melanization reactions to form the blackened nodules (Lavine and Strand, 2002). Moreover, there may be a cooperative effect of these early bacterial metabolites for inducing effective immunosuppression, which would facilitate *X. nematophila* bacterial growth. Seo and Kim (2011) showed that a mixture of bacterial metabolites is more potent for suppressing immune responses than individual compounds at the same concentration. By considering the longer culture period of *X. nematophila* in TSB medium compared to the growth time in insect body, these bacterial metabolites must have been synthesized and released into insect hemocoel within 6 h after bacterial infection. Thus, the inhibitory activities of PHPP and Ac-FGV against PO and PLA_2 lead to the suppression of phagocytosis and nodulation during the early infection stage of the bacteria in insect hosts.

After 12 h in TSB culture, all metabolites were detected in the culture broth and were effective at suppressing *S. exigua* immune responses. Thus, the exponential growth (12–18 h) of the *X. nematophila* population in infected *S. exigua* is explained by the massive production of bacterial metabolites. Hemocytes underwent significant cell death during this exponential phase. This hemocytic activity can be explained by the production of BZA and PY, which were produced during late bacterial growth and were highly cytotoxic at low concentrations. Indeed, these metabolites accumulated to 58.8 μg/ml (BZA) and 133.6 μg/ml levels, which was above their I_{50} cytotoxic levels of 2.25 μg/ml and 1.75 μg/ml. Thus, the significant decrease in the hemocyte population at the late infection stage (12–18 h in insects) was due to the *X. nematophila* cytotoxic bacterial metabolites. In contrast to non-protein substances, protein cytotoxins have been reported in *X. nematophila*. A hemolysin, XhlA, has been identified in *X. nematophila* that lyses granulocytes and plasmatocytes of *Manduca sexta* (Cowles and Goodrich-Blair, 2005). Another toxin called Xax (= α-xenorhabdolysin) was identified from *X. nematophila* and triggers apoptosis of insect and mammalian cells (Vigneux *et al*., 2007). The cytotoxic effect of BZA and PY may be due to their strong cellular PLA_2 ($cPLA_2$) inhibitory activity. $cPLA_2$ is required for cell survival in vertebrate systems, and secretory PLA_2 ($sPLA_2$) is

associated with this activity (Costa-Junior *et al*., 2006; Cinque *et al*., 2008). Thus, BZA and PY may selectively induce hemocyte lysis. This speculation needs to be clarified in a future study after molecular identification of $cPLA_2$ in insects. Until now, only $sPLA_2$ has been identified at the molecular level in insects (Shrestha *et al*., 2010).

Eicosanoids are immune mediators in insects and inhibition of their synthesis leads to significant immunosuppression (Kim *et al*., 2005). Our analysis of eight bacterial metabolites with respect to the temporal biosynthesis and release pattern indicated that oxindole was the initial *X. nematophila* bacterial metabolite. Oxindole was inhibitory against *S. exigua* PLA_2 activity, suggesting that eicosanoid synthesis should be inhibited as the first step in bacterial infection for successful pathogenicity. After suppression of eicosanoid biosynthesis, other bacterial metabolites were synthesized and released to induce various pathogenic activities, such as inhibition of PO activity and hemocyte nodulation as well as toxicity of hemocytes.

In summary, *X. nematophila* sequentially synthesized eight secondary metabolites exhibiting different inhibitory activities against various immune-associated processes in target insects. At the early infection phase, *X. nematophila* synthesized metabolites that were highly inhibitory against PO and PLA_2 enzyme activities to suppress acute cellular immunity. In late phase, the bacteria synthesized cytotoxic compounds that induced hemocyte lysis and effectively suppressed immune responses. Moreover, there may be cooperative activities among the bacterial metabolites to induce host immunosuppression.

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