Journal of Asia-Pacific Entomology 23 (2020) 449–457

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full length article

Benzylideneacetone and other phenylethylamide bacterial metabolites induce apoptosis to kill insects

Md. Mahi Imam Mollah, Fatema Yeasmin, Yonggyun Kim*

Department of Plant Medicals, College of Life Sciences, Andong National University, Andong 36729, Republic of Korea

ARTICLE INFO

Keywords:
Cytotoxicity
Xenorhabdus
Photorhabdus
Benzylideneacetone
Bacterial metabolite
Apoptosis
Insecticide

ABSTRACT

Xenorhabdus and *Photorhabdus* are entomopathogenic bacteria that can induce immunosuppression against target insects by suppressing eicosanoid biosynthesis, leading to fatal septicemia. These bacteria can synthesize and release secondary metabolites such as benzylideneacetone (BZA) and other phenylethylamide compounds that can inhibit phospholipase A_2 (PLA_2) and shut down eicosanoid biosynthesis. However, insecticidal activities of these bacterial metabolites remain unclear. Thus, the objective of this study was to assess cytotoxicities of BZA and seven other bacterial metabolites to insect cells. These eight bacterial metabolites exhibited significant cytotoxicities against an insect cell line Sf9 at micromolar range. Especially, BZA and cPY were highly potent at low micromolar range. When these eight bacterial metabolites were injected to hemocoels of *Spodoptera exigua* larvae, they significantly decreased total count of hemocytes. In Sf9 cell line and hemocytes, these bacterial metabolites induced cell membrane blebbings, apoptotic vesicles, and genomic DNA fragmentation. Terminal deoxyribonucleotidyl transferase nick end translation assay showed that these bacterial metabolites caused significant DNA breakages in cells in a dose-dependent manner. However, a pan caspase inhibitor treatment significantly rescued the cell death induced by these bacterial metabolites. Cytotoxicities of these bacterial metabolites were highly correlated with their insecticidal activities. These results indicate that the insecticidal activities of the bacterial metabolites may be induced by their apoptotic activities against hemocytes and other insect cells. Taken together, these results suggest that phenylethylamide compounds might have potential as novel insecticides.

Introduction

Two entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus*, exhibit mutual symbiosis with *Steinernema* and *Heterorhabditis*, respectively (Akhurst, 1980; Boemare, 2002). These bacteria reside in the intestine of host nematode. Although their host nematodes are in dauer larval form, they can infect target insects (Kaya and Gaugler, 1993). Once the infective juvenile (IJ) nematodes enter insect hemocoels through natural openings such as mouth, anus, or spiracles, they can release their symbiotic bacteria (Forst and Clarke, 2002). In the hemocoel, these bacteria can release immunosuppressive factors that mostly target phospholipase A_2 (PLA_2) to shutdown eicosanoid biosynthesis (Park and Kim, 2000).

Xenorhabdus and *Photorhabdus* are likely to produce a variety of bacterial secondary metabolites based on their biosynthetic gene clusters (BGCs) such as genes involved in non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) (Tobias et al., 2017). These bacterial metabolites can suppress insect immunity. For example, rhabducin is an isocyanide-containing compound produced from a BGC consisting of *isnA*, *isnB*, and a glycosyltransferase and can inhibit the activity of phenoloxidase (PO) in the wax moth, *Galleria mellonella* (Crawford et al., 2012). More than 70 kinds of rhabdopeptide/xenortide peptides (RXPs) have been derived from BGCs encoding multiple NRPS subunits. They share structural similarities with protease inhibitors, suggesting that RXPs can suppress PO activation presumably by inhibiting serine proteases (Cai et al., 2016; Sussmuth and Mainz, 2017). Some bacterial metabolites such as phurealipids are products of NRPS/PKS. They could inhibit juvenile hormone (JH) degradation, enhance JH levels, and prevent expression of antimicrobial peptide genes (Nollmann et al., 2015). Benzylideneacetone (BZA), a bacterial metabolite of *X. nematophila* (Ji et al., 2004), can inhibit PLA_2 and prevent eicosanoid biosynthesis (Vatanparast et al., 2019). Later, Seo et al. (2012) have identified other PLA_2 inhibitors from bacterial metabolites of *X. nematophila* and *P. temperata temperata*. Eicosanoids are a group of oxygenated C20 polyunsaturated fatty acids (PUFAs) derived from arachidonic acid (AA) (Stanley, 2000). They can mediate both

* Corresponding author.
E-mail address: hosanna@anu.ac.kr (Y. Kim).

https://doi.org/10.1016/j.aspen.2020.03.008
Received 10 February 2020; Received in revised form 12 March 2020; Accepted 16 March 2020
Available online 18 March 2020
1226-8615/ © 2020 Korean Society of Applied Entomology. Published by Elsevier B.V. All rights reserved.

cellular and humoral immune responses against various microbial pathogens in insects (Kim et al., 2018). PLA_2 can catalyze the committed step of eicosanoid biosynthesis to release precursors such as AA and other PUFAs for eicosanoid biosynthesis (Hasan et al., 2019a). Furthermore, PLA_2 activity can be induced by pathogen infection (Tunaz et al., 2003). Inhibition of PLA_2 activity by bacterial metabolites of *Xenorhabdus* and *Photorhabdus* can lead to immunosuppression of target insects (Eom et al., 2014a).

Bacterial infection by *Xenorhabdus* and *Photorhabdus* can kill insects within 24 h (Park and Kim, 2000; Kang et al., 2004). Such insecticidal activity can be explained by septicemia and toxemia induced by these bacteria (Tobias et al., 2017). Several toxin proteins have been identified in these bacteria, including Xpt (Morgan et al., 2001), XnGroEL (Kumari et al., 2014), Txp40 (Brown et al., 2006), XaxAB (Vigneux et al., 2007), a 12 kDa protein (Hemalatha et al., 2018), and PirAB (Yang et al., 2017). In addition, several secondary metabolites produced from PKS-NRPS catalytic activities have been proposed to contribute to bacterial pathogenicity by inducing immunosuppression and toxemia (Shi and Bode, 2018). In the meantime, bacterial culture broth of *X. nematophila* or *P. temperata temperata* is cytotoxic to hemocytes of a lepidopteran insect, *Spodoptera exigua* (Cho and Kim, 2004). Seo et al. (2012) have demonstrated that BZA and other phenylethylamide bacterial metabolites possess insecticidal activity. However, little is known about the mode of action involved in the insecticidal activity of the bacterial secondary metabolites.

This study hypothesized that BZA and other bacterial metabolites could induce apoptosis of hemocytes and other insect cells to kill target insects. To test this hypothesis, this study assessed their cytotoxicities to Sf9 cells and hemocytes of *S. exigua* using an *in vitro* cell viability test and an *in vivo* cytotoxicity test, respectively. Subsequently, the cytotoxicity was assessed by apoptosis such as morphological changes of target cells, DNA fragmentation test, and terminal deoxyribonucleotidyl transferase nick end translation (TUNEL) assay. To support the apoptosis induced by the bacterial metabolites, a rescue test was performed using an apoptosis inhibitor. Finally, the correlation between the insecticidal activity and apoptosis-inducing activity of bacterial metabolites was determined.

Materials and methods

Insect rearing

S. exigua used in this study was originated from a field population infesting welsh onion, Andong, Korea. Its larvae were reared under laboratory conditions (25 ± 1 °C, 16:8h of L: D photoperiod, and 60 ± 5% relative humidity) with an artificial diet (Goh et al., 1990). Adults were fed 10% sucrose solution. Larval instars (L1-L5) were determined based on their head capsule sizes described by Goh et al. (1990). L5 larvae were collected from cohorts for experimental purpose.

Sf9 cell culture

Sf9 cell line (IPLB-Sf21-AE) was derived from *Spodoptera frugiperda* pupal ovarian tissue and obtained from Professor Yeon-Ho Je (Seoul National University, Seoul, Korea). Sf9 cells were cultured in TC-100 insect cell culture medium (Welgene, Daegu, Korea) containing 10% heat-inactivated fetal bovine serum (Welgene). Cells were maintained in 25 cm^2 tissue culture flask (Nunc, Roskilde, Denmark) at 28 °C and split every 4–5 days.

Chemicals

Benzylideneacetone (BZA), *p*-hydroxyphenylpropionic acid (PHPP), 2-oxindole, 4-hydroxyphenylacetic acid (HPA), and indole were purchased from Sigma-Aldrich Korea (Seoul, Korea) and dissolved in dimethyl sulfoxide (DMSO). Acetylated phenylalanine-glycine-valine (Ac-FGV), proline-tyrosine (PY), and *cyclo*-proline-tyrosine (cPY) were chemically synthesized by Peptron (Daejon, Korea). MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) and a caspase inhibitor Z-valine-alanine-aspartic acid fluoromethyl ketone (Z-VAD-FMK) were purchased from Sigma-Aldrich Korea. Anticoagulant buffer (ACB, pH 4.5) was prepared with 186 mM NaCl, 17 mM Na_2EDTA, and 41 mM citric acid. Phosphate-buffered saline (PBS, pH 7.4) was prepared to contain 100 mM phosphoric acid.

Cytotoxicity based on MTT assay

MTT assay was performed following the method described by Boonsuepsakul et al. (2008) using Sf9 cells. Briefly, Sf9 cells were seeded into 96-well plates at a density of 1.2×10^4 cells per well and cultured for 24 h at 28 °C. Cells were then treated with various concentrations (0.01, 0.1, 1, 10, and 100 μM) of bacterial metabolites (BMs) and incubated for another 24 h at 28 °C. After adding 10 μL of MTT (5 mg/mL in PBS) to each well, cells were cultured at 28 °C for 4 h. Purple formazan granules formed by viable cells were dissolved in 50 μL DMSO. The absorbance was then measured at 570 nm using a microplate reader (Victor Multi-label Plate Reader, PerkinElmer, Waltham, MA, USA).

Cytotoxicity based on total hemocyte count (THC)

To assess hemolytic activity of BMs, L5 larvae were injected with BMs at different doses (0.0003, 0.003, 0.03, 0.3, 3 μg/larva). DMSO was injected as control. After incubating at room temperature (RT) for 24 h, the hemolymph was collected and diluted with ACB to count THC using a hemocytometer (Superior Marienfeld, Lauda-Konigshofen, Germany). Each treatment was replicated three times. Each replication represented a hemolymph sample from individual larva randomly chosen from each treatment group.

Apoptosis based on cell morphology

Cell membrane blebs (see a photo in Fig. 3A) were indicators of apoptosis-like symptom. Sf9 cells were seeded into 12-well plates at a density of 2×10^4 cells per well and cultured for 24 h at 28 °C. These cells were then treated with BMs at different concentrations (0.001, 0.01, 0.1, 10, and 100 μM) and incubated at 28 °C for another 24 h. The number of blebbing cells wad then counted among randomly chosen 100 cells under an inverted microscope (CKX31, Olympus, Tokyo, Japan) at 400× magnification.

Apoptosis based on DNA fragmentation assay

DNA fragmentation assay was performed using Sf9 cells to observe the characteristic internucleosomal cleavage of DNA during apoptosis known to lead to DNA laddering pattern (Goswami et al. 1999). Briefly, Sf9 cells (2×10^6 cells) were treated with BMs (BZA and cPY) at different concentrations and cultured at 28 °C for 48 h. These cells were then harvested and disrupted in 500 μL lysis buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.2% Triton X-100) on ice for 20 min. Fragmented DNA was separated from nuclei by centrifuging at 5000 × *g* for 10 min. DNA fragments containing supernatants were incubated at 50 °C for 4 h, purified using phenol:chloroform:isoamyl alcohol (25:24:1), and separated on 1.5% agarose gel.

Terminal deoxyribonucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed using an *in situ* Cell Death Detection kit (Abcam, Cambridge, UK) and hemocytes of L5 larvae. Briefly, a hemocyte suspension was prepared using bleeding hemolymph obtained

from 5 ~ 6 L5 larvae diluted in ~700 μL of ACB. After incubating on ice for 40 min, the hemocyte suspension was replaced with TC100. A reaction mixture consisted of 10 μL of hemocyte suspension, 1 μL of 10 μM 5-bromouridine (BrdU) solution containing TdT, and 1 μL of bacterial metabolite. The mixture was then placed on a cover glass in a wet chamber. After 18 h of incubation at 4 °C, the medium was replaced with 2% paraformaldehyde and incubated at 4 °C for 10 min. Cells were washed with PBS, added with 0.3% Triton-X in PBS, and incubated for 5 min at RT. After blocking with 5% bovine serum albumin in PBS for 10 min, cells were incubated with mouse anti-BrdU antibody (diluted 1:15 in blocking solution) for 1 h at RT. After washing out unbound anti-BrdU antibody, cells were incubated with FITC-conjugated anti-mouse IgG antibody (diluted 1:300 in blocking solution) for 1 h. After washing with PBS, 10 μL DAPI was added followed by incubation at RT for 5 min. Cells were then washed with 10 μL PBS. Glycerol: PBS solution (10 μL) was then added to cells on a cover glass which was then placed onto a glass slide for observation under a fluorescence microscope (DFC450 C, Leica, Wetzlar, Germany) in FITC mode. To rescue apoptosis, a pan caspase inhibitor (Z-VAD-FMK) was co-injected at a concentration (1 μg/larva) along with BMs to L5 larvae. Subsequently, TUNEL assay was performed followed the method described above. Apoptosis rate was scored by counting FITC-signalling cells among randomly chosen 100 cells. Each treatment was replicated three times. Each replication represented individual hemolymph collection from randomly chosen larvae in each treatment.

Insecticidal bioassays

To determine toxicities of BMs, three different bioassays (hemocoelic injection, topical application on dorsal surface, and leaf-dipping method for oral administration) were performed. For hemocoelic injection, a 10 μL microsyringe (Hamilton, Reno, NV, USA) was used to inject 2 μL of BMs into L5 larvae. Treated larvae were individually reared to prevent cannibalism. For topical application, 0.5 μL test sample was applied onto L2 larvae. For oral administration, 2 cm^2 cabbage leaf was dipped into BM solution and provided to L2 larvae. Treated larvae with hemocoelic injection or topical application were placed in 9 cm diameter dishes and incubated at 25 ± 2 °C with a relative humidity of 60 ± 10%. In the same conditions, oral administration used 24 h feeding of treated leaves. Each test concentration used 10 larvae per replication. Each treatment was replicated three times. Mortality was scored every 24 h. The toxicity analysis used mortality data at 96 h after treatment. Larvae were considered dead by an absence of movement upon touching. Larvae treated with solvent (DMSO) used to dissolve BMs were used as controls.

Data analysis

All data for continuous variables were subjected to one-way analysis of variance (ANOVA) using PROG GLM in SAS program (SAS Institute, 1989). Mortality data were subjected to arcsine transformation and used for ANOVA. Means were compared with the least significant difference (LSD) test at Type I error (P value) = 0.05. Median lethal dose (LD_{50}) and median lethal concentration (LC_{50}) for cytotoxicity and insecticidal activity were subjected to Probit analysis using EPA Probit Analysis Program, ver. 1.5 (U.S. Environmental Protection Agency, USA).

Results

Cytotoxicity of bacterial metabolites (BMs)

Eight BMs were exposed to Sf9 cells. Their cytotoxicities to cells were monitored using MTT assay (Fig. 1). With increasing concentration of BM used for treatment, Sf9 cells showed significant (F = 1924.11; df = 5, 96; P < 0.0001) impairment of MTT

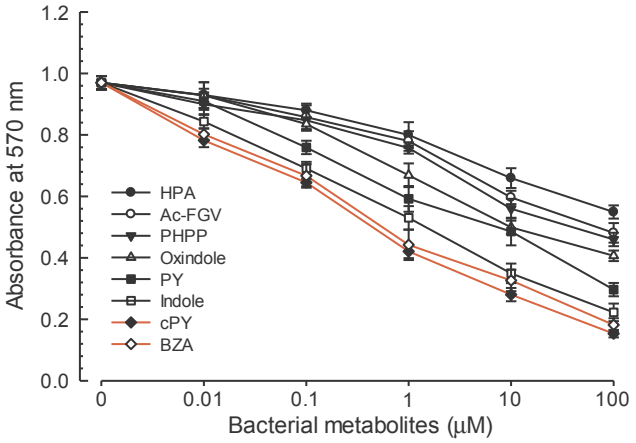

Fig. 1. Cytotoxicities of eight bacterial metabolites (BMs) against Sf9 cells assessed by MTT assay. After Sf9 cells were seeded into 96-well plates (1.2×10^4 cells per well), they were allowed to adhere and grow for 24 h at 28 °C. Then these cells were exposed to different concentrations of BMs and incubated for another 24 h at the same temperature. After 4 h of incubation with MTT, formed formazan granules were dissolved in DMSO and measured by absorbance at 570 nm. Each treatment was replicated three times. Error bars represent standard deviations.

metabolism in a dose-dependent manner. Although cytotoxicities were observed for all eight BMs, cPY and BZA were more potent than others (F = 1034.96; df = 1, 96; P < 0.0001). LC_{50} of BMs against Sf9 cells ranged from 0.42 to 6.45 μM (Table 1). Of these BMs, cPY and BZA had the lowest LC_{50} values.

Cytotoxicities of these BMs against Sf9 cells suggested that they might have hemolytic activities against *S. exigua* hemocytes. To test this hypothesis, they were injected to larval hemocoels of *S. exigua* to assess their cytotoxicities against hemocytes (Fig. 2). Total number of hemocytes of control larvae was counted at about 1.7×10^7 cells/mL. At 24 h post-injection, the total count of hemocytes was significantly (F = 1211.10; df = 5, 96; P < 0.0001) reduced in a dose-dependent manner for all tested BMs. LD_{50} of BMs against hemocytes ranged from 0.37 to 4.35 μg/larva (Table 1). BZA and cPY exhibited the highest cytotoxicities (F = 36.98; df = 1, 96; P < 0.0001) against *S. exigua* hemocytes. In contrast, PHPP, Ac-FGV, and HPA exhibited low (< 50%) toxicities even at the highest treated dose.

Bacterial metabolites can induce apoptotic cell death

To explain cytotoxicities of BMs, changes in cell structure were assessed after exposing Sf9 cells to BMs (Fig. 3). Compared to control Sf9 cells, cells treated with BZA at 0.1 μM exhibited cellular membrane blebs and small vesicles around cells (Fig. 3A). The number of cells exhibiting blebs was significantly (F = 3623.79; df = 5, 96; P < 0.0001) increased with increasing concentration of BMs used for treatment (Fig. 3B). Especially, cPY and BZA exhibited the most potent (F = 165.21; df = 1, 96; P < 0.0001) effects in inducing cell blebs.

Exposure of Sf9 cells to two potent BMs (BZA and cPY) resulted in DNA fragmentation (Fig. 4). Control cells showed a single genomic band at > 10 kb region. In contrast, cells exposed to the BMs exhibited laddering patterns of their genomic DNAs at regions < 1 kb. Such DNA fragmentation was dependent on exposure concentrations of the BMs. After treatment over 0.01 μM, DNA laddering patterns were evident.

TUNEL assay supported that DNA fragmentation was induced by BMs (Fig. 5). Hemocytes were exposed to BMs and broken DNA ends were labelled with BrdU and TdT. Labelled BrdU was detected with a FITC-labelled antibody. FITC labelling was detected in the nucleus stained with DAPI. As expected, few FITC signal was detected in control hemocytes (Fig. 5A). Although all tested BMs significantly (F = 127.97; df = 7, 16; P < 0.0001) induced DNA fragmentation, cPY and BZA

Table 1
Comparative analyses of cytotoxicities and insecticidal activities of eight bacterial metabolites.

Bacterialmetabolites	Cytotoxicity		
	MTT assay LC_{50} ± SE (μM)	THC[1] assay LD_{50} ± SE (μg)	Apoptosis assay[2] LC_{50} ± SE (μM)
BZA	0.48 ± 0.03	0.37 ± 0.15	1.13 ± 0.64
cPY	0.42 ± 0.02	0.46 ± 0.09	1.78 ± 0.03
Indole	0.89 ± 0.01	0.58 ± 0.09	2.38 ± 0.60
PY	2.01 ± 0.04	2.11 ± 0.19	4.80 ± 1.56
Oxindole	1.04 ± 0.01	1.98 ± 0.12	3.28 ± 1.39
PHPP	3.92 ± 0.03	3.18 ± 0.02	5.55 ± 1.19
Ac-FGV	5.01 ± 0.02	3.90 ± 0.25	8.46 ± 0.62
HPA	6.45 ± 0.01	4.35 ± 0.21	14.02 ± 0.74
	Insecticidal activity		
	Injection LD_{50} ± SE (μg)	Topical application LD_{50} ± SE (μg)	Feeding assay LC_{50} ± SE (ppm)
BZA	0.02 ± 0.42	0.18 ± 0.45	2.81 ± 0.57
cPY	0.04 ± 0.46	0.56 ± 0.51	7.98 ± 0.54
Indole	0.44 ± 0.42	0.83 ± 0.50	16.20 ± 0.50
PY	0.44 ± 0.43	0.248 ± 0.47	115.31 ± 0.64
Oxindole	0.11 ± 0.45	1.39 ± 0.53	38.95 ± 0.55
PHPP	0.39 ± 0.44	1.25 ± 0.49	139.72 ± 0.75
Ac-FGV	0.94 ± 0.45	4.16 ± 0.49	493.75 ± 0.70
HPA	1.45 ± 0.49	9.26 ± 0.53	947.36 ± 0.64

[1] Total hemocyte count
[2] Apoptosis was assessed with TUNEL assay.

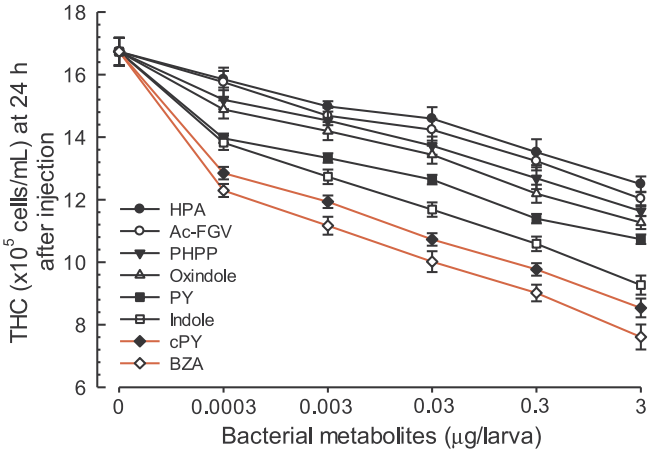

Fig. 2. Cytotoxicities of bacterial metabolites (BMs) against hemocytes of *S. exigua*. L5 larvae were injected with different concentrations of BMs. DMSO was injected as control. After 24 h incubation at room temperature, the hemolymph was collected to determine total hemocyte count (THC). Each treatment was replicated three times. Error bars represent standard deviations.

were significantly (F = 615.38; df = 1, 16; P < 0.0001) more potent than other BMs (Fig. 5B). LC_{50}s of these BMs based on cytotoxicity measured by TUNEL assay ranged from 1.13 to 14.02 μM (Table 1). cPY and BZA had low micromolar LC_{50}s against hemocytes.

Caspase inhibitor can rescue cytotoxicity caused by bacterial metabolites

Cell membrane blebs and DNA fragmentation suggested that the cytotoxicity induced by BMs might be explained by cell apoptosis. Caspases are known to play crucial role in mediating apoptosis (Denton et al., 2013). To determine whether the cytotoxicity induced by BMs might be explained by cell apoptosis, a pan caspase inhibitor (Z-VAD-FMK) was added to BM treatments to rescue cytotoxicity (Fig. 6). As expected, cytotoxicities of BMs against hemocytes were significantly (F = 13.39; df = 1, 20; P < 0.0016) reversed by the addition of an

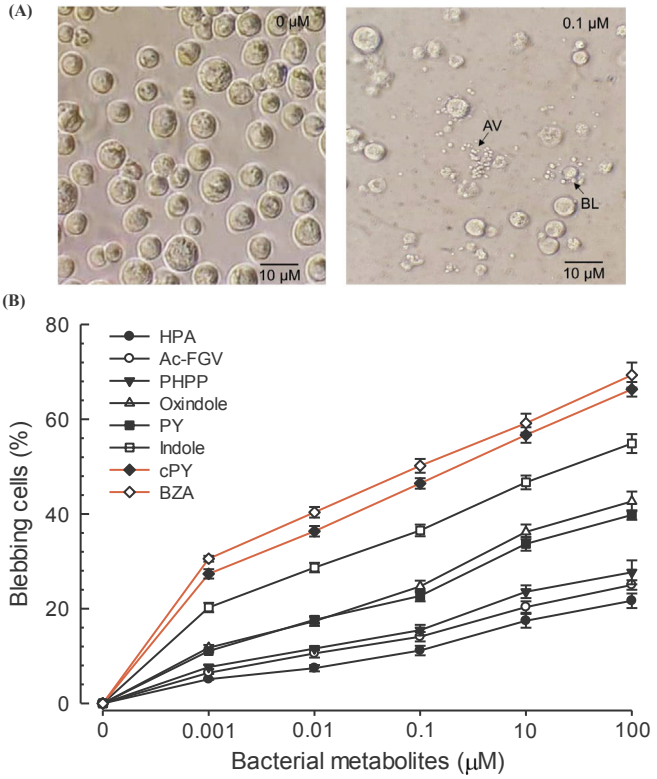

Fig. 3. Formation of apoptotic vesicles (AVs) of Sf9 cells after exposure to each bacterial metabolite (BM). (A) Death symptoms of Sf9 cells after exposure to 0.1 μM of BZA. 0 μM represents DMSO solvent control. 'BL' represents blebbing cell membrane. (B) Quantitative analysis of apoptotic Sf9 cells using BL symptom. Sf9 cells were seeded into 12-well plates at density of 2 × 10^4 cells per well and incubated at 28 °C for 24 h. These cells were then exposed to different doses of BMs and incubated for another 24 h. The number of blebbing cells were counted among randomly chosen 100 cells under an inverted microscope. Each treatment was replicated three times. Error bars represent standard deviations.

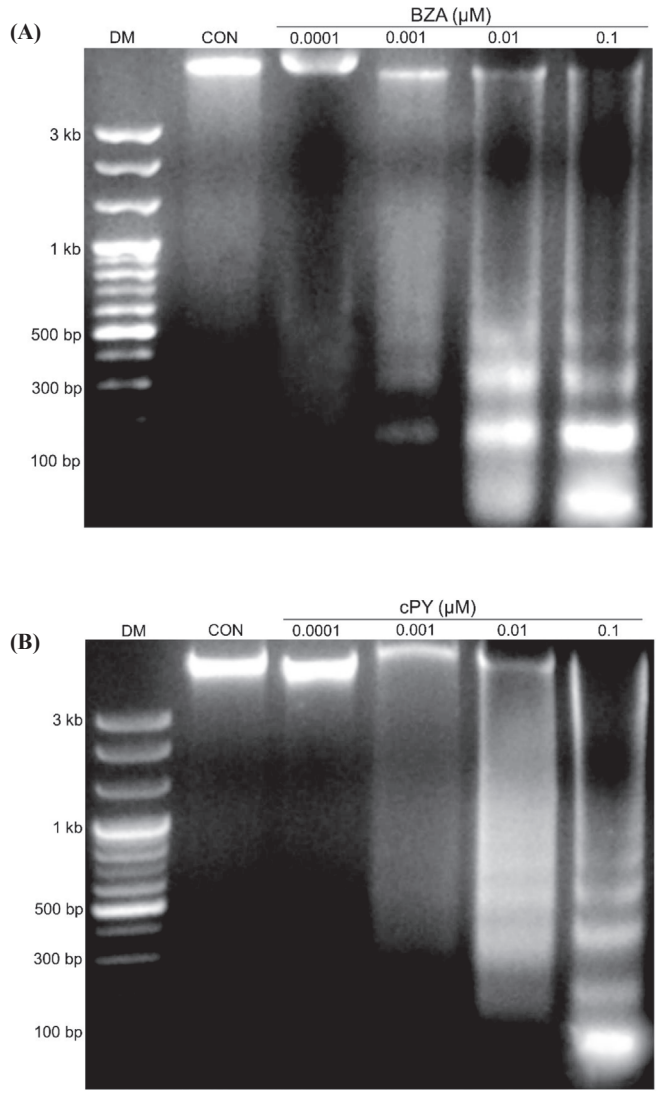

Fig. 4. DNA fragmentation of Sf9 cells after exposure to two bacterial metabolites (BZA and cPY). Sf9 cells were seeded into 6-well plates and incubated at 28 °C for 24 h. These cells were then treated with different concentrations of BZA or cPY and incubated for another 48 h at the same temperature. DMSO was used in treatment as control ('CON'). Cells were then harvested to extract genomic DNA. Fragmented genomic DNAs were separated on 1.5% agarose gel. 'DM' represents DNA size marker.

apoptosis inhibitor. For cPY and BZA, the rescue effect was increased depending on the amount of the caspase inhibitor added.

Bacterial metabolites exhibit significant insecticidal activity

Cytotoxicities of these BMs suggested that they might possess insecticidal activities. To test this hypothesis, these metabolites were injected to larval hemocoels of *S. exigua* (Fig. 7A). For injection assay, we used L5 larvae to effectively deliver 2 μL volume of BM test solution into the internal body. All eight BMs exhibited significant ($F = 33.84$; df = 7, 128; $P < 0.0001$) insecticidal activities. Of them, BZA and cPY were more potent ($F = 177.10$; df = 1, 128; $P < 0.0001$) than other BMs. They killed test insects at low concentrations (Table 1). Interestingly, these BMs also had oral and contact toxicities against *S. exigua* larvae. In these assay, we used L2 larvae, which might be susceptible to feeding or topical assays. Topical application of these BMs resulted in significant ($F = 22.26$; df = 7, 128; $P < 0.0001$) mortalities, especially when BZA and cPY ($F = 46.28$; df = 1, 128; $P < 0.0001$) were used for treatments (Fig. 7B). However, their LD_{50} values were much higher than those when they were injected (Table 1). Feeding BMs also resulted in significant ($F = 30.79$; df = 7, 128; $P < 0.0001$) mortalities (Fig. 7C). BZA and cPY had low LC_{50} values (much < 10 ppm) (Table 1).

Based on toxicity data, correlation analysis was performed using cytotoxicity data of different BMs exhibiting differential potencies (Table S1). Cytotoxicity measured by MTT assay had high correlations ($r = 0.90 \sim 0.98$; $P < 0.05$) with LD_{50}s or LC_{50}s measured in insecticidal activities. Similarly, their cytotoxicities measured by THC change or apoptosis also had high correlations ($r = 0.84 \sim 0.97$; $P < 0.05$) or ($r = 0.89 \sim 0.98$; $P < 0.05$) with their insecticidal activities.

Discussion

Xenorhabdus and *Photorhabdus* have great potential in producing secondary metabolites that can alter physiological processes of insects because of their obligate mutualism with entomopathogenic nematodes (Tobias et al., 2017). This is supported by their genome composition consisting a number of predicted BGCs including NRPS and PKS (Chaston et al., 2011). The present study was performed to determine insecticidal activities of eight secondary metabolites derived from bacterial culture broth of *Photorhabdus* and *Xenorhabdus*. Seo et al. (2012) have shown that the bacterial metabolites can inhibit PLA_2, thus suppressing insect immune responses. However, it was difficult to explain their acute insecticidal activities by the inhibition of PLA_2 enzyme activity. Thus this study was conducted to focus on cytotoxic effects of the bacterial metabolites on insect cells.

All eight bacterial metabolites exhibited potent cytotoxicities against insect cells such as Sf9 and hemocytes. When *S. exigua* larvae are infected with *X. nematophila*, they undergo fatal hemolysis (Park and Kim, 2000). Park et al. (2005) have shown that an organic extract of *X. nematophila* culture broth can lead to apoptosis-like cell death of *S. exigua* hemocytes. All eight compounds have been identified from the bacterial culture broth of *X. nematophila* (Ji et al., 2004; Seo et al., 2012). This study showed that their individual compounds had cytotoxic potencies against insect cells. Among these metabolites, BZA and cPY exhibited the highest cytotoxicities against insect cells. These two BMs are potent in inhibiting PLA_2 enzyme activity. They also exhibit antibacterial activities (Ji et al., 2004; Wattana-Amorn et al., 2016). These two compounds share chemical structure of phenylethylether (benzene–CH_2–CH_2–CH = O) which is similar to phenylethylamide (benzene–CH_2–CH_2–CH = O and –NH–). Phenylethylamide is a core structure that can inhibit subtype 7 serotonin receptor of *S. exigua* (Hasan et al., 2019b). This suggests that BZA and cPY might induce cytotoxicity by inhibiting serotonin signal.

All eight bacterial metabolites induced apoptosis of hemocytes which underwent DNA fragmentation and suffered cell membrane blebs. The genome DNA in cells exposed to metabolites showed DNA laddering patterns presumably due to DNase action between nucleosomes. DNA fragmentation was further supported by TUNEL assay, a method for detecting DNA fragmentation by labelling 3′–OH termini at DNA breaks generated during apoptosis (Gorczyca et al., 1993). In insects, apoptosis plays an important role in many cellular processes including development, tissue homeostasis, and innate antiviral response (Miura, 2011). A wide range of internal and external stimuli including DNA damage, viral infection, and growth factor can induce apoptosis of target cells (Cho and Choi, 2002). Most apoptotic reactions include the activation of caspases (cysteine protease cleaving after aspartic acid) (Cohen, 1997). However, AIF (apoptosis-inducing factor), which locates in the intermembrane of mitochondria, is released upon apoptosis signal and translocated into nucleus where it increases chromatin condensation and DNA fragmentation by unknown mechanisms (Susin et al., 1999). Caspases are produced in apo-enzyme form. They can be activated through proteolysis into two large and small subunits, then

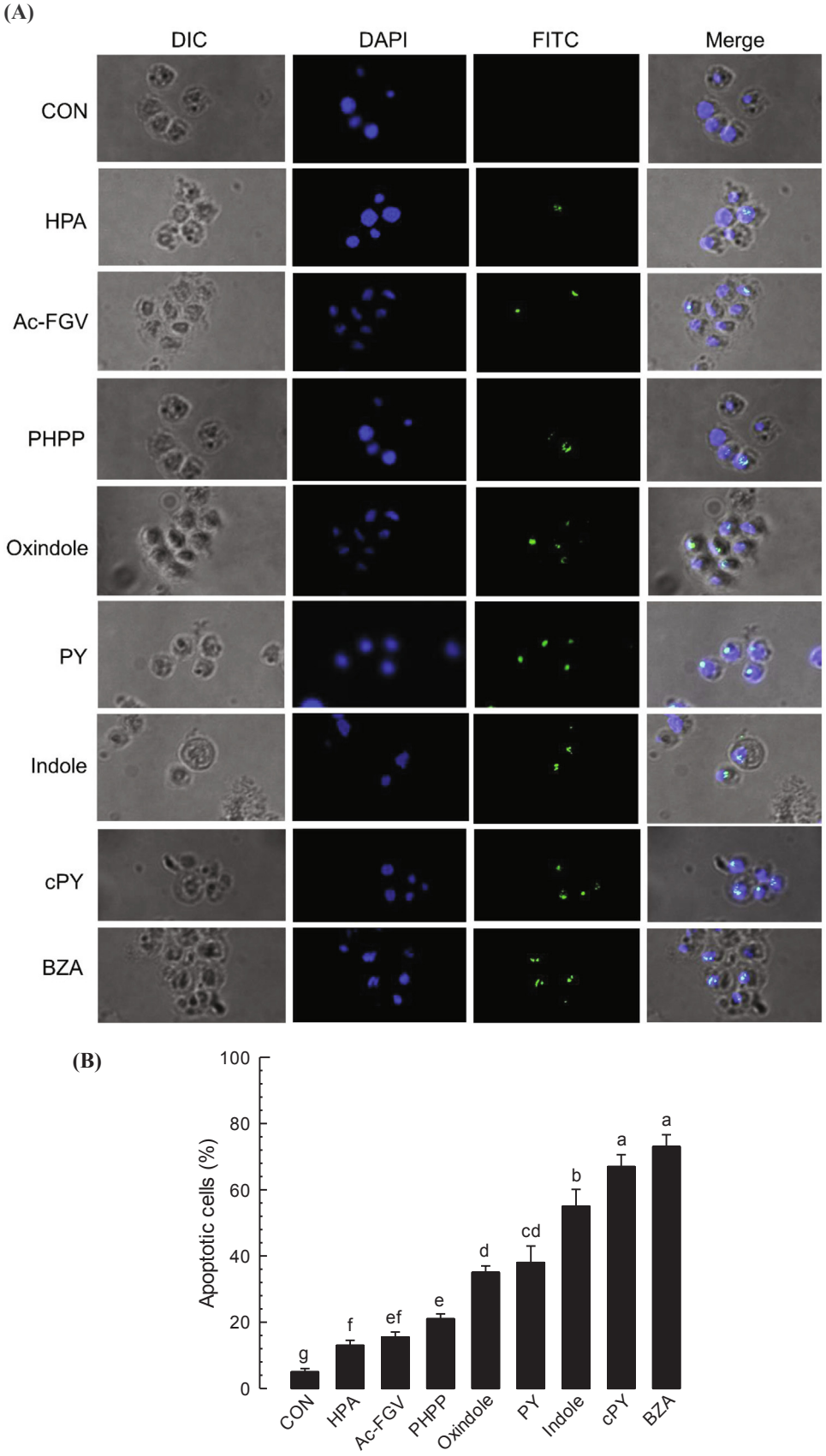

Fig. 5. TUNEL assay of hemocytes collected from L5 larvae of *S. exigua* after exposure to different bacterial metabolites (BMs) at concentration of 10 μM. (A) DNA fragments visualized by TUNEL after exposure to BMs. Hemocytes were collected from larvae treated with BMs and labelled with 5-bromouridine (BrdU) solution containing TdT. Then specific antibody against BrdU was added to specifically bind to labelled DNA. The antibody complex was then detected with secondary antibody conjugated with FITC. Nuclei were stained with DAPI. Cells were observed under a fluorescence microscope at 400× magnification. (B) Scoring apoptotic hemocytes exposed to BMs at concentration of 10 μM by counting the number of FITC-signalling cells among randomly chosen 100 cells. Each treatment was replicated three times. Error bars represent standard deviations.

forming a tetrameric active form from two molecules of pro-enzymes (Kumar and Colussi, 1999). Treatment with a pan caspase inhibitor, z-VAD-FMK, significantly reversed cytotoxicities of BMs, supporting the notion that the cytotoxicity was induced by the apoptosis. A previous study has shown that FAD-glucose dehydrogenase (GLD) is a prime signal of apoptosis induction of *S. exigua* hemocytes in response to

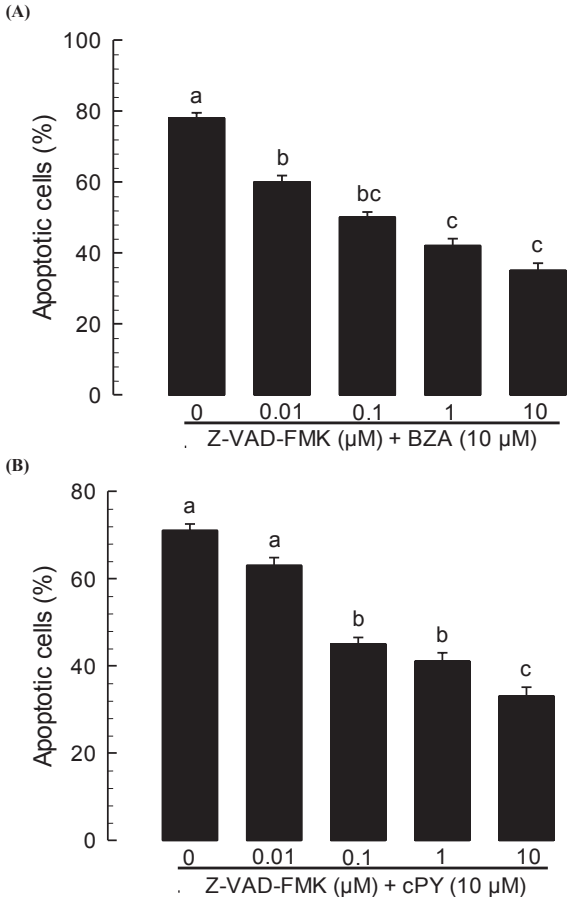

Fig. 6. Rescue effect of a pan caspase inhibitor (Z-VAD-FMK) on hemocytes undergoing apoptosis after exposure to cytotoxic BZA (A) or cPY (B). Different concentrations of the inhibitor were co-injected along with 10 μM concentration of bacterial metabolites to L5 larvae of *S. exigua*. Subsequent TUNEL assay scored apoptosis rates. Each replication represented individual hemolymph collection from randomly chosen larvae in each treatment. Each treatment was replicated three times. Error bars represent standard deviations.

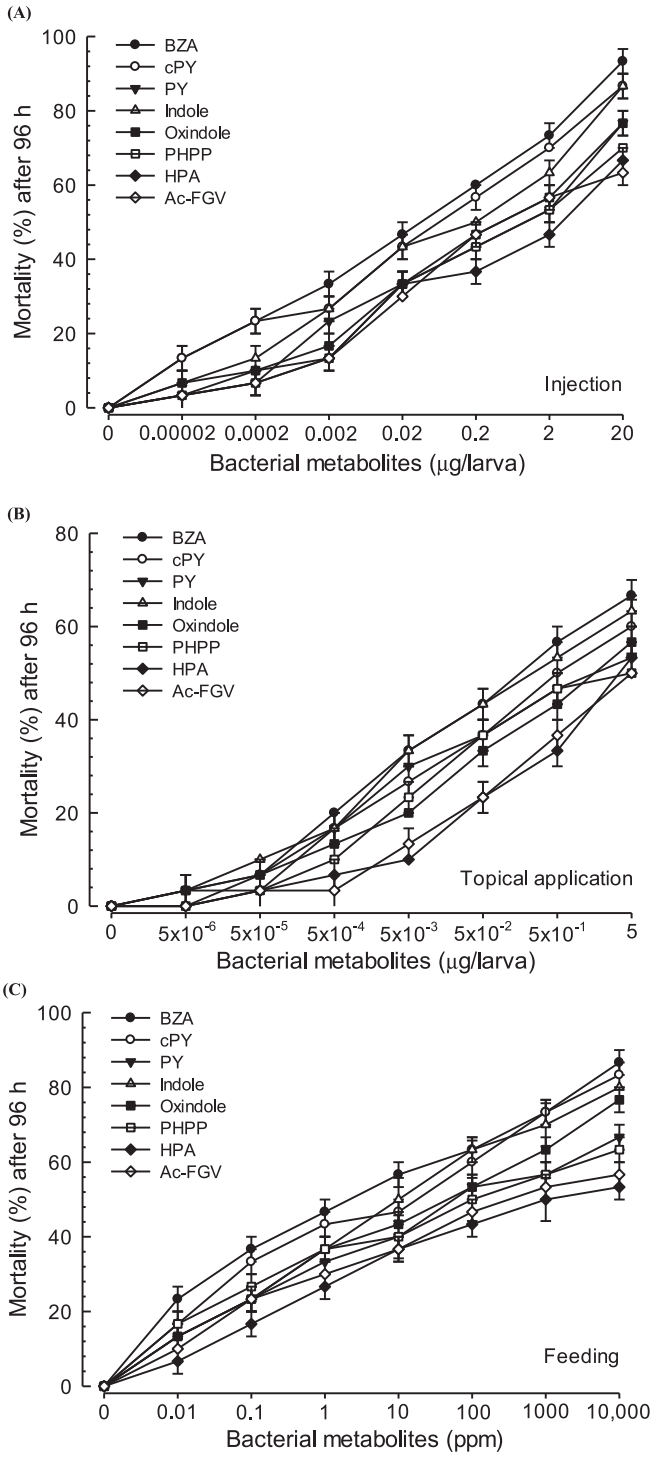

Fig. 7. Insecticidal toxicities of eight different bacterial metabolites (BMs) against larvae of *S. exigua*. (A) Hemocoelic injection with 2 μL of different concentrations of BMs into L5 larvae. (B) Contact poisoning by topical application on dorsal surface of L2 larvae with 0.5 μL test BMs. (C) Oral toxicity by leaf-dipping method. Feeding to L2 larvae used 2 cm^2 cabbage leaf soaked in different concentrations of BM solutions. Treated larvae were placed in 9 cm diameter dishes and incubated at 25 ± 2 °C and a relative humidity of 60%. Mortality was recorded at 96 h after treatment. DMSO solvent were used as control to treat larvae. Each treatment used 10 larvae and replicated three times. Error bars represent standard errors.

infection by *X. nematophila* (Park et al., 2005). GLD is associated with the production of reactive oxygen species which is a main inducer of apoptosis (Lovallo et al., 2002). On the other hand, GLD plays a crucial role in mediating cellular immune responses in insects (Cox-Foster and Stehr, 1994). GLD is present in plasmatocytes and granules of granular cells. However, its activity is only detected in immune-activated plasmatocytes (Cox-Foster and Stehr, 1994). GLD can catalyze the oxidation of glucose to gluconolactone and participate in immune reactions by donating electrons during regeneration of its cofactor FAD. These electrons can be transferred to quinines as electron acceptors (Lovallo and Cox-Foster, 1999), resulting in superoxide anion radicals that are highly reactive (Cox-Foster and Stehr, 1994) and required for hemocyte encapsulation (Lovallo and Cox-Foster, 1999). Thus, the GLD activity is required for activating cellular immune response of hemocytes before undergoing apoptosis. In this regard, BZA and cPY may activate GLD to induce inappropriate apoptosis of hemocytes and other cells, leading to fatal cytotoxicity. This is supported by an observation in a human cancer cell line ('U937'), in which BZA can induce apoptosis of by activating caspase-3 (Hwang et al., 2003). However, a direct functional link between BZA and GLD remains still unknown.

Bacterial secondary metabolites tested in the present study had insecticidal activities when they were administered orally or by contact. A previous study has shown that the bacterial metabolites can act as insecticidal synergists of other bacterial pathogens by suppressing target insects' immune responses. For example, a bacterial culture broth can enhance bacterial pathogenicity of *Bacillus thuringiensis* (Eom et al., 2014b). This study showed that cytotoxicities of the bacterial secondary metabolites were highly correlated with their insecticidal activities.

Production of bacterial secondary metabolites responsible for insecticidal activity is regulated by a global transcriptional factor Lrp (leucine responsive regulatory protein) in *X. nematophila* (Casanova-Torres et al., 2017). This virulence modulation is caused by variations of Lrp protein level in the bacteria. Bacteria with low level of Lrp can become virulent (Hussa et al., 2015). In contrast, *P. luminescens* uses another regulator, Hfq, which regulates gene expression at post-transcription in bacteria (Butland et al., 2005), for the production of the secondary metabolites (Tobias et al., 2017). Eom et al. (2014a) have shown that the eight BMs tested in the present study might be sequentially synthesized in insect hemocoels after infection. Thus, bacterial secondary metabolites produced under the control of regulators after *Xenorhabdus* or *Photorhabdus* can monitor external environment in insect hemocoels and exhibit their cytotoxicities by inducing apoptosis for successful parasitization of host nematodes.

CRediT authorship contribution statement

Md. Mahi Imam Mollah: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Fatema Yeasmin:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization. **Yonggyun Kim:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by a grant (No. 2017R1A2B3009815) of National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning (MSIP), Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2020.03.008.

References

Akhurst, R.J., 1980. Morphological and functional dimorphism in *Xenorhabdus* spp. Bacteria symbiotically associated with the insect pathogenic nematodes *Neoplectana* and *Heterorhabditis*. J. Gen. Microbiol. 121, 303–309.

Boemare, N.E., 2002. Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus*. In: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI, New York, pp. 5–56.

Boonsuepsakul, S., Luepromchai, E., Rongnoparut, P., 2008. Characterization of *Anopheles minimus* CYP6AA3 expressed in a recombinant baculovirus system. Arch. Insect Biochem. Physiol. 69, 13–21.

Brown, S.E., Cao, A.T., Dobson, P., Hines, E.R., Akhurst, R.J., East, P.D., 2006. Txp40, a Ubiquitous insecticidal toxin protein from *Xenorhabdus* and *Photorhabdus* bacteria. Appl. Environ. Microbiol. 72, 1653–1662.

Butland, G., Peregrín-Alvarez, J.M., Li, J., Yang, W., Yang, X., Canadien, V., Starostine, A., Richards, D., Beattie, B., Krogan, N., Davey, M., Parkinson, J., Greenblatt, J., Emili, A., 2005. Interaction network containing conserved and essential protein complexes in *Escherichia coli*. Nature 433, 531–537.

Cai, X., Nowak, S., Wesche, F., Bischoff, I., Kaiser, M., Furst, R., Bode, H.B., 2016. Entomopathogenic bacteria use multiple mechanisms for bioactive peptide library design. Nat. Chem. 9, 379–386.

Casanova-Torres, Á.M., Shokal, U., Morag, N., Eleftherianos, I., Goodrich-Blair, H., 2017. The global transcription factor Lrp is both essential for and inhibitory to *Xenorhabdus nematophila* insecticidal activity. Appl. Environ. Microbiol. 31, 83.

Chaston, J.M., Suen, G., Tucker, S.L., Andersen, A.W., Bhasin, A., Bode, E., Bode, H.B., Brachmann, A.O., Cowles, C.E., Cowles, K.N., Darby, C., de Léon, L., Drace, K., Du, Z., Givaudan, A., Herbert Tran, E.E., Jewell, K.A., Knack, J.J., Krasomil-Osterfeld, K.C., Kukor, R., Lanois, A., Latreille, P., Leimgruber, N.K., Lipke, C.M., Liu, R., Lu, X., Martens, E.C., Marri, P.R., Médigue, C., Menard, M.L., Miller, N.M., Morales-Soto, N., Norton, S., Ogier, J.C., Orchard, S.S., Park, D., Park, Y., Qurollo, B.A., Sugar, D.R., Richards, G.R., Rouy, Z., Slominski, B., Slominski, K., Snyder, H., Tjaden, B.C., van der Hoeven, R., Welch, R.D., Wheeler, C., Xiang, B., Barbazuk, B., Gaudriault, S., Goodner, B., Slater, S.C., Forst, S., Goldman, B.S., Goodrich-Blair, H., 2011. The entomopathogenic bacterial endosymbionts *Xenorhabdus* and *Photorhabdus*: convergent lifestyles from divergent genomes. PLoS One 6, e27909.

Cho, S.G., Choi, E.S., 2002. Apoptotic signaling pathway: caspases and stress-activated protein kinases. J. Biochem. Mol. Biol. 35, 24–27.

Cho, S., Kim, Y., 2004. Hemocyte Apoptosis Induced by Entomopathogenic Bacteria, *Xenorhabdus* and *Photorhabdus*, in *Bombyx mori*. J. Asia Pac. Entomol. 7, 195–200.

Cohen, J.J., 1997. Caspases: The executioners of apoptosis. Biochem. J. 326, 1–16.

Cox-Foster, D.L., Stehr, J.E., 1994. Induction and localization of FAD-glucose dehydrogenase (GLD) during encapsulation of abiotic implants in *Manduca sexta* larvae. J. Insect Physiol. 40, 235–249.

Crawford, J.M., Portmann, C., Zhang, X., Roeffaers, M.B., Clardy, J., 2012. Small molecule perimeter defense in entomopathogenic bacteria. Proc. Natl. Acad. Sci. USA 109, 10821–10826.

Denton, D., Aung-Htut, M.T., Kumar, S., 2013. Developmentally programmed cell death in *Drosophila*. Biochim. Biophys. Acta 1833, 3499–3506.

Eom, S., Park, Y., Kim, H., Kim, Y., 2014b. Development of a high efficient "Dual Bt-Plus" insecticide using a primary form of an entomopathogenic bacterium, *Xenorhabdus nematophila*. J. Microbiol. Biotechnol. 24, 507–521.

Eom, S., Park, Y., Kim, Y., 2014a. Sequential immunosuppressive activity of bacterial secondary metabolites from the entomopathogenic bacterium *Xenorhabdus nematophila*. J. Microbiol. 52, 161–168.

Forst, S., Clarke, D., 2002. Bacteria–nematode symbiosis. In: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI Publishing, New York, pp. 57–77.

Goh, H.G., Lee, S.G., Lee, B.P., Choi, K.M., Kim, J.H., 1990. Simple mass-rearing of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), on an artificial diet. Korean J. Appl. Entomol. 29, 180–183.

Gorczyca, W., Traganos, F., Jesionowska, H., Darzynkiewicz, Z., 1993. Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells. Analogy to apoptosis of somatic cells. Exp. Cell Res. 207, 202–205.

Goswami, J., Sinskey, A.J., Steller, H., Stephanopoulos, G.N., Wang, D.I.C., 1999. Apoptosis in batch cultures of Chinese hamster ovary cells. Biotechnol. Bioeng. 62, 633–640.

Hasan, M.A., Ahmed, S., Kim, Y., 2019a. Biosynthetic pathway of arachidonic acid in *Spodoptera exigua* in response to bacterial challenge. Insect Biochem. Mol. Biol. 111, 1–15.

Hasan, A., Yeom, H.S., Ryu, J., Bode, H.B., Kim, Y., 2019b. Phenylethylamides derived from bacterial secondary metabolites specifically inhibit an insect serotonin receptor. Sci. Rep. 9, 20358.

Hemalatha, D., Prabhu, S., Rani, W.B., Anandham, R., 2018. Isolation and characterization of toxins from *Xenorhabdus nematophilus* against *Ferrisia virgata* (Ckll.) on tuberose. *Polianthes tuberosa*. Toxicon. 146, 42–49.

Hussa, E.A., Casanova-Torres, Á.M., Goodrich-Blair, H., 2015. The global transcription factor Lrp controls virulence modulation in *Xenorhabdus nematophila*. J. Bacteriol. 197, 3015–3025.

Hwang, S.Y., Paik, S., Park, S.H., Kim, H.S., Lee, I.S., Kim, S.P., Baek, W.K., Suh, M.H., Kwon, T.K., Park, J.W., Park, J.B., Lee, J.J., Suh, S.I., 2003. N-phenethyl-2-phenylacetamide isolated from *Xenorhabdus nematophilus* induced apoptosis through caspases activation and calpain-mediated Bax cleavage in U937 cells. Int. J. Oncol. 22, 151–157.

SAS Institute Inc., 1989. SAS/STAT user's guide, Release 6.03, Ed. Cary, NC.

Ji, D., Yi, Y., Kim, G.H., Choi, Y.H., Kim, P., Baek, N.I., Kim, Y., 2004. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. FEMS Microbiol. Lett. 239, 241–248.

Kang, S., Han, S., Kim, Y., 2004. Identification of an entomopathogenic bacterium, *Photorhabdus temperata* subsp. *temperata*, in Korea. J. Asia Pac. Entomol. 7, 331–337.

Kaya, H.K., Gaugler, R., 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38, 1–206.

Kim, Y., Ahmed, S., Stanley, D., 2018. Eicosanoid-mediated immunity in insects. Dev Comp Immunol. 83, 130–143.

Kumar, S., Colussi, P.A., 1999. Prodomains-adaptors-oligomerization: the pursuit of caspase activation in apoptosis. Trends Biochem. Sci. 24, 1–4.

Kumari, P., Kant, S., Zaman, S., Mahapatro, G.K., Banerjee, N., Sarin, N.B., 2014. A novel insecticidal GroEL protein from *Xenorhabdus nematophila* confers insect resistance in tobacco. Transgenic Res. 23, 99–107.

Lovallo, N., Cox-Foster, D.L., 1999. Alteration in FAD-glucose dehydrogenase activity and hemocyte behavior contribute to initial disruption of *Manduca sexta* immune response to *Cotesia congregata* parasitoids. J. Insect Physiol. 45, 1037–1048.

Lovallo, N., Mcpheron, B.A., Cox-Foster, D.L., 2002. Effects of the polydnavirus of *Cotesia congregata* on the immune system and development of non-habitual hosts of the parasitoid. J. Insect Physiol. 48, 517–526.

Miura, M., 2011. Active participation of cell death in development and organismal homeostasis. Develop. Growth Differ. 53, 125–136.

Morgan, J.A.W., Sergeant, M., Ellis, D., Ousley, M., Jarrett, P., 2001. Sequence analysis of insecticidal genes from *Xenorhabdus nematophilus* PMFI296. Appl. Environ. Microbiol. 67, 2062–2069.

Nollmann, F.I., Heinrich, A.K., Brachmann, A.O., Morrisseau, C., Mukherjee, K., Casanova-Torres, A.M., Kleinhans, D., Kinski, S., Schultz, K., Beeton, M., Kaiser, M., Chu, Y.Y., Ke, L.P., Thanwisai, A., Bozhuyuk, K.A.J., Chantratita, N., Gotz, F., Waterfield, N.R., Vilcinskas, A., Stelzer, E.H.K., Goodrich-Blair, H., Hammock, B.D.,

Bode, H.B., 2015. A *Photorhabdus* natural product inhibits insect juvenile hormone epoxide hydrolase. Chembiochem. 16, 766–771.
Park, Y., Choi, Y., Kim, Y., 2005. An entomopathogenic bacterium, *Xenorhabdus nematophila*, causes hemocyte apoptosis of beet armyworm *Spodoptera exigua*. J. Asia Pac. Entomol. 8, 153–159.
Park, Y., Kim, Y., 2000. Eicosanoids rescue *Spodoptera exigua* infected with *Xenorhabdus nematophila*, the symbiotic bacteria to the entomopathogenic nematode *Steinernema carpocapsae*. J. Insect Physiol. 46, 1469–1476.
Seo, S., Lee, S., Hong, Y., Kim, Y., 2012. Phospholipase A_2 inhibitors synthesized by two entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus temperata* spp. *temperata*. Appl. Environ. Microbiol. 78, 3816–3823.
Shi, Y.M., Bode, H.B., 2018. Chemical language and warfare of bacterial natural products in bacteria-nematode-insect interactions. Nat. Prod. Rep. 35, 309–335.
Stanley, D.W., 2000. Eicosanoids invertebrate signal transduction systems. Princeton University Press, Princeton, NJ.
Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Constantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., Kroemer, G., 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397, 441–446.
Sussmuth, R.D., Mainz, A., 2017. Nonribosomal peptide synthesis-principles and prospects. Angew. Chem. Int. Ed. Engl. 56, 3770–3821.
Tobias, N.J., Wolff, H., Djahanschiri, B., Grundmann, F., Kronenwerth, M., Shi, Y.M., Simonyi, S., Grün, P., Shapiro-Ilan, D., Pidot, S.J., Stinear, T.P., Ebersberger, I., Bode, H.B., 2017. Natural product diversity associated with the nematode symbionts *Photorhabdus* and *Xenorhabdus*. Nat. Microbiol. 2, 1676–1685.
Tunaz, H., Park, Y., Buyukguzel, K., Bedick, J.C., Nor Aliza, A.R., Stanley, D.W., 2003. Eicosanoids in insect immunity: Bacterial infection stimulates hemocytic phospholipase A_2 activity in tobacco budworms. Arch. Insect Biochem. Physiol. 52, 1–6.
Vatanparast, M., Ahmed, S., Sajjadian, S.M., Kim, Y., 2019. A prophylactic role of a secretory PLA_2 of *Spodoptera exigua* against entomopathogens. Dev. Comp. Immunol. 95, 108–117.
Vigneux, F., Zumbihl, R., Jubelin, G., Ribeiro, C., Poncet, J., Baghdiguian, S., Givaudan, A., Brehelin, M., 2007. The xaxAB genes encoding a new apoptotic toxin from the insect pathogen *Xenorhabdus nematophila* are present in plant and human pathogens. J. Biol. Chem. 282, 9571–9580.
Wattana-Amorn, P., Charoenwongsa, W., Williams, C., Crump, M.P., Apichaisataienchote, B., 2016. Antibacterial activity of cyclo (L-Pro-L-Tyr) and cyclo (D-Pro-L-Tyr) from Streptomyces sp. strain 22–4 against phytopathogenic bacteria. Nat. Prod. Res. 30, 1980–1983.
Yang, Q., Zhang, J., Li, T., Liu, S., Song, P., Nangong, Z., Wang, Q., 2017. PirAB protein from *Xenorhabdus nematophila* HB310 exhibits a binary toxin with insecticidal activity and cytotoxicity in *Galleria mellonella*. J. Invertebr. Pathol. 148, 43.