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Development of a High Efficient "Dual Bt-Plus" Insecticide Using a Primary Form of an Entomopathogenic Bacterium, *Xenorhabdus nematophila*

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A phase variation has been reported in an entomopathogenic bacterium, *Xenorhabdus nematophila*. Compared with a wild-type primary form, a secondary form usually loses several physiological and biochemical characters. This study showed that the phase variation of *X. nematophila* caused a significant alteration in its immunosuppressive activity and subsequent entomopathogenicity. A secondary form of *X. nematophila* was detected in laboratory colonies and exhibited significant differences in dye absorption and entomopathogenicity. In addition, the secondary form was different in its production of eicosanoid-biosynthesis inhibitors (EBIs) compared with the primary form of *X. nematophila*. Production of oxindole and *p*-hydroxypropionic acid was significantly reduced in the culture broth of the secondary form of *X. nematophila*. The reduced EBI production resulted in significant suppression in the inhibitory effects on cellular nodule formation and phenoloxidase activity. Culture broth of the primary form of *X. nematophila* enhanced the pathogenicity of *Bacillus thuringiensis* (Bt) significantly more than the culture broth of the secondary form. Furthermore, this study developed a highly efficient "Dual Bt-Plus" to control both lepidopteran insect pests *Plutella xylostella* and *Spodoptera exigua*, by mixing two effective Bt strains along with the addition of potent bacterial metabolites or 100-fold concentrated *X. nematophila* culture broth.

Keywords: Insecticide, immune response, benzylideneacetone, oxindole, *Bacillus thuringiensis*, *Xenorhabdus nematophila*

Introduction

The entomopathogenic bacterium *Xenorhabdus nematophila* is mutualistic to a nematode, *Steinernema carpocapsae* [50]. The infective juveniles (IJs) of *S. carpocapsae* enter the hemocoel of target insects through natural openings, such as the mouth, anus, or spiracle [33]. In the hemocoel, IJs release *X. nematophila* from their gut and suppress target insect immunity [10]. After the bacterial growth, the host nematodes begin to grow and reproduce in the insect cadaver after the bacterial septicemia [15]. The multiplied nematodes become the IJs and reassociate with *X. nematophila* and come out of the insect cadavers to look for other target insects [28].

Insects defend the bacterial infection with their innate immune responses [3]. Insect immunity is divided into cellular and humoral responses. Cellular immunity is the response mostly exhibited by hemocytes, such as phagocytosis and nodulation in response to bacterial infection [35]. Humoral immunity consists of the chemical reactions of the antimicrobial peptides depending on bacterial cell wall patterns [37]. The acute antibacterial responses are executed by cellular immunity [22]. The successful pathogenity of *X. nematophila* is partially explained by its inhibitory activity against the insect immunity. Lipopolysaccharide of *X. nematophila* inhibits phenoloxidase

activity and is cytotoxic to insect hemocytes [10, 11]. Indeed, a toxin (α-Xenorhabdolysis) of *X. nematophila* induces apoptosis in both insect and mammalian cells [66]. In terms of immunosuppression by *X. nematophila*, Park and Kim [44] proposed a hypothesis that the bacteria inhibit eicosanoid biosynthesis, because it has been well known that eicosanoids play crucial roles in reducing insect immune responses [63]. Eicosanoids are a group of C20 oxygenated and polyunsaturated fatty acids [61]. Specifically, eicosanoid derived from both cyclooxygenase (COX) and lipoxygenase (LOX) products mediate insect immunity signals to induce both cellular and humoral responses [29]. In eicosanoid biosynthesis, arachidonic acid (AA) release from a phospolipid is a crucial committed step and then is catalyzed by phospholipase A_2 (PLA_2), because AA is the major substrate for COX and LOX [62]. Both entomopathogenic bacterial groups, *Xenorhabdus* and *Photorhabdus*, inhibit PLA_2 to suppress eicosanoid biosynthesis and immune responses of target insects [31]. Indeed, Seo *et al*. [54] identified seven PLA_2 inhibitiors from the bacterial culture broth of *X. nematophila*.

A "Bt-Plus" insecticide has been developed to enhance the insecticidal activity of *Bacillus thuringiensis* (Bt) by suppressing target insect immunity by the culture broth of *X. nematophila* [53]. The synergistic effect of Bt and *X. nematophila* on the insecticidal activity was initially confirmed by their independent targets of the midgut and the hemocoel, in which the midgut epithelium was disintegrated by Bt and the hemocoel was exposed to *X. nematophila* [26]. Then, *X. nematophila* suppresses the target insect immunity against the invasion of Bt and other microbes, which ultimately enhances the Bt pathogenicity [27, 52]. Similar synergism of *X. nematophila* to Bt was also confirmed in the Mediterranean flour moth, *Ephestia kuehniella*, in which only Cry toxins with high binding affinity to the insect midgut epithelium were effective to enhance the Bt toxicity [4]. In the meantime, a variant of *X. nematophila* was found in the successively cultured colonies. Our current variant no longer absorbs bromothymol blue dye and exhibits a low insecticidal activity at hemocoelic injection. Owing to a well-known phase variation in *X. nematophila* [5], the variant was suspected to be a secondary form of *X. nematophila*. This study compared the primary form (newly isolated bacteria from an entomopathogenic nematode, *S. carpocapsae*) with the secondary form in terms of pathogenicity, PLA_2 inhibitor biosynthesis, and immunosuppression. Then this study developed a highly efficient Bt-Plus insecticide against both *Plutella xylostella* and *Spodoptera exigua*, by mixing two effective Bt strains and the culture broth of the primary form of *X. nematophila*.

Materials and Methods

Rearing Insects

S. exigua larvae were reared in a laboratory under the conditions of 25 ± 1ºC and 16 h of light : 8 h of darkness. The larvae were fed with artificial diet [18], whereas adults were fed 10% sucrose solution. The final (fifth) instar larvae were used for bioassay and hemolymph collection in this study. *P. xylostella* larvae were reared with cabbage at the same rearing environment. The final (fourth) instar larvae were used for the insecticide bioassay.

Isolation of the Primary Form of *X. nematophila*

Infective nematodes (IJs) of *S. carpocapsae* Pochon were topically applied on the fifth instar larvae of *S. exigua* and incubated at 25°C for 48 h. The infected hosts were surface-sterilized with 70% ethanol, and the first abdominal prolegs were cut by a pair of sterile scissors to collect the exuded hemolymph. The hemolymph was diluted with an equal volume of sterilized water and streaked on tryptic soy agar (Difco, Sparks, MD, USA) to isolate bacteria. The isolated bacteria were cultured in Luria-Bertani medium (LB, Difco) at 25°C for 48 h. After washing the cultured cells three times with sterilized water by centrifuging the culture medium at 4,000 ×*g* for 2 min at 4°C, the cells were resuspended in sterilized insect Ringer's solution [23] for further experiments. Another primary form of *X. nematophila* was obtained from the American Type Culture Collection (ATCC 101061; Manassas, VA, USA). The secondary form analyzed in this study was originally isolated from *S. carpocapsae* [25] and successively cultured *in vitro*.

Biolog Identification System

Isolates were prepared according to the manufacturer's instruction in the OmniLog ID System User Guide (Biolog, Hayward, CA, USA). Each isolate was cultured on tryptic soy broth (TSB) at 28°C for 48 h. Each cultured isolate was emulsified to the specific density in the inoculation fluid (0.40% sodium chloride, 0.03% Pluronic F-68, and 0.02% Gellan Gum). Each well of the GN Microplate (Biolog) was subsequently inoculated with 150 µl of the bacterial suspension and incubated at 28°C for 24 h. Each metabolic profile was compared with the appropriate GN Omnilog Biolog database (Biolog) and used to identify the bacterial species.

HPLC Analysis of PLA_2 Inhibitor from the Bacterial Culture Broth

For analysis of bacterial metabolites using HPLC, 1 L of *X. nematophila* was cultured in LB for 48 h at 28ºC. After removing bacterial cells by centrifugation at 8,000 rpm for 20 min, the supernatant was mixed with the same volume of hexane in a separate funnel. The organic extract was concentrated using an evaporator. The dried extract was resuspended with methanol and analyzed by HPLC (Waters, Milford, MA, USA). Samples were cleaned with a PTFE syringe filter (Cronus, Churcham, UK).

Ten milliliters of the cleaned sample was injected to an HPLC equipped with a C18 column (Deltapak, 15 mm, 300 A, 300 × 7.8 mm). The samples were then separated with a mobile phase of methanol:water (60:40 (v/v)) at a flow rate of 0.5 ml/min for 30 min with a UV detector (Waters, Milford, MA, USA) at 254 nm. The eight bacterial metabolites included oxindole, indole, *p*-hydroxypropionic acid (PHPP), cyclo-Pro-Tyr (cPY), 4-hydroxyphenylacetic acid (HPA), benzylideneacetone (BZA), Pro-Tyr (PY), and acetylated Phe-Gly-Val (Ac-FGV).

Bioassay of *X. nematophila* by Hemocoelic Injection

Test bacteria of *X. nematophila* were cultured as described above. Final instar larvae (fourth instar of *P. xylostella* and fifth instar of *S. exigua*) were surface-sterilized with 70% ethanol. The bacterial culture medium (10^3–10^5 CFU/ml) was injected into the insects by Hamilton microsyringe (Hamilton, Reno, Nevada, USA). Each treatment consisted of three replicates, and each replicate used 30 insect larvae. Control larvae were injected with sterile Ringer's solution. Mortality was assessed at 24 h after the bacterial injection. Dead larvae were determined by no voluntary movement after prodding test larvae with a blunt stick.

Bioassay of "Bt-Plus"

Test solutions of Bt-Plus were prepared by mixing Bt and *X. nematophila* culture broths. Bacterial culture broth (48 h at 28°C in TSB) of *X. nematophila* was serially diluted with the uncultured fresh TSB medium containing 1,000 μg/ml of Bt. Bt insecticides used in this study were *B. thuringiensis* ssp. *kurstaki* (Btk) and *B. thuringiensis* ssp. *aizawai* (Bta) used in the earlier studies [53, 54]. The Bt suspensions were kept at 4°C for 2 days to form spores. Finally, the spore densities of Btk and Bta were 7.4×10^{10} and 8.7×10^8 CFU/ml, respectively. Cabbage leaves were soaked for 10 min in different Bt-Plus suspensions and dried under darkness for 30 min. Each treatment was replicated three times. Each replication used 10 larvae of *P. xylostella* (fourth instar) or *S. exigua* (fifth instar). At every 24 h, the number of the live individuals was counted for 7 days.

Bioassay of "Dual Bt-Plus"

For Dual Bt-Plus, two Bt strains were mixed before adding to the culture broth of *X. nematophila*. Two Bt strains were mixed with a 4:1 (v/v) ratio of Bta and Btk. The first type of Dual Bt-Plus ("Bt-Plus+oxindole" or "Bt-Plus+BZA") was prepared by the additional oxindole or BZA at 100 ppm to the Bt-Plus spraying solution (0.5% Bt mixture, 0.4% *X. nematophila* culture broth, and 0.1% ethanol). The other type of Dual Bt-Plus was prepared with the Bt-Plus spraying solution including a 100-fold high concentration of *X. nematophila* by addition of the bacterial culture broth (0.5% Bt mixture, 40% *X. nematophila* culture, and 0.1% ethanol). The bioassay followed the method as described above.

Phenoloxidase (PO) Activity

Hemolymph PO activity was determined using L-3,4-dihydroxyphenylalanine (DOPA) as a substrate [30]. Hemolymph was collected into a 1.5 ml tube by cutting the abdominal proleg. The sample solution consisted of 1 μl of test material with 9 μl of hemolymph. The PO substrate solution prepared had 990 μl of 100 mM phosphate-buffered saline (0.7% NaCl, pH 7.4) containing 20 μg/μl of DOPA in acetone. The PO reaction was initiated by adding the sample solution to the PO substrate solution. The initial absorbance change was monitored at 495 nm in a spectrophotometer.

PLA_2 Activity

Hemocyte PLA_2 activity was fluorometrically determined with a substrate, the pyrene-labeled phospholipids [1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycerol-3-phosphatidyl choline] in the presence of bovine serum albumin (BSA), as described by Radvanyi *et al*. [47]. The fluorescent phospholipid was prepared at 0.2 mM concentration using ethanol. For preparation of enzyme source, hemolymph was collected into a 1.5 ml tube containing a few granules of phenylthiourea and centrifuged at 400 ×*g* for 3 min. The plasma was removed and washed three times with washing buffer (50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA). The hemocyte pellet was resuspended in the washing buffer and homogenized by an ultrasonicator (Bandelin Sonoplus, Berlin, Germany) for 10 min at 3 cycles and 75% power. Protein concentrations of hemocyte extracts were measured by the Bradford method [6] using BSA as a standard. The reaction mixture was prepared in a 96-well microplate by adding 142.5 μl of Tris buffer, 1 μl of 1 M $CaCl_2$, 1 μl of 10% BSA, and 2 μl of 0.2 mM substrate. The sample solution consisted of 1 μl of test material with 1 μl of hemocyte enzyme extract and was incubated for 20 min at room temperature. The PLA_2 reaction was initiated by adding the sample solution (2 μl) to the reaction mixture (146.5 μl). The fluorescence intensity was monitored with an Aminco Bowmen Series 2 luminescence spectrometer (FA257; Spectronic Instruments, Madison, WI, USA) using excitation and emission wavelengths of 345 and 394 nm, respectively. The enzyme activity was calculated in pmol/min using a formula provided by Radvanyi *et al*. [47].

Nodulation Assay

The nodulation assay was performed by injecting 10^4 cells of *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) in 2 μl, through the abdominal proleg, using a microsyringe, into hemocoel of *S. exigua* as previously described [45]. After 8 h incubation at room temperature, test insects were dissected and counted in melanized nodules under a stereoscopic microscope (SZX9; Olympus, Tokyo, Japan). For the nodulation inhibition assay, 2 μl of different *X. nematophila* bacterial concentrations was injected into each larva after *E. coli* injection, and nodules were counted as described above.

Statistical Analysis

Survival data were transformed by the square root and arcsine method for normalization. Treatment means and variances of the

transformed data were analyzed by PROC GLM of the SAS program [51].

Results

Comparison of Primary and Secondary Forms of *X. nematophila* in Physiological and Biochemical Characters

A laboratory colony ("Xnk2") of *X. nematophila* exhibited a low entomopathogenic activity. A hemocoelic injection of 10^3 CFU of *X. nematophila* gave only 60–70% mortality to *S. exigua* larvae at 24 h. In a previous study, the same dose of *X. nematophila* would kill 100% of the identical age of *S. exigua* [44]. To explain this decrease of insecticidal activity of *X. nematophila*, we needed to compare the laboratory colony with a primary form of *X. nematophila*. One was to use a primary type strain ("ATCC" strain) of *X. nematophila*. The other was to isolate a new *X. nematophila* ("Xnk1") from the hemolymph of the larvae infected with the nematode IJs. In a similar way to the ATCC strain, Xnk1 exhibited a blue colony on NBTA medium due to absorption of bromothymol blue dye. However, the Xnk2 colony did not show the blue colony. To compare the biochemical properties of carbon source utility of the two forms of *X. nematophila*, the bacteria were cultured on Biolog plate and the outcomes were compared (Table 1). Xnk1 resulted in 99% identity with respect to the carbon utility characters of the canonical *X. nematophila* described in the Biolog database system. However, Xnk2 characters showed only 54% identity with those of *X. nematophila*, even though the 16S rDNA sequence of XnK2 perfectly (100%) matched to that of the *X. nematophila* K1 strain [25] originally isolated from the nematode host.

Comparison of Primary and Secondary Forms of *X. nematophila* in PLA_2 Inhibitor Production

Eight bacterial secondary metabolites of *X. nematophila* were reported to inhibit insect PLA_2 [24, 54]. All compounds were extracted in hexane extracts (Fig. 1). In both primary forms of Xnk1 and ATCC strains of *X. nematophila*, all eight metabolites were detected; however, the secondary form of Xnk2 produced the compounds at significantly lower quantities than the two primary forms, and oxindole and BZA were not detected in the secondary form (Table 2). There was little difference between the two primary forms in the production of eight secondary metabolites.

Comparison of Primary and Secondary Forms of *X. nematophila* in Immunosuppression and Entomopathogenicity

The decrease of PLA_2 inhibitor synthesis in the secondary

Table 1. Comparison of variants of *X. nematophila* with respect to carbon utility.

No.	Carbon sources for bacterial growth	Xn[1]	Xnk1[2]	Xnk2[3]
1	Water	-	-	-
2	α-Cyclodextrin	-	-	-
3	Dextrin	+	+	-
4	Glycogen	+	+	+
5	Tween 40	-	-	+
6	Tween 80	+	+	-
7	*N*-Acetyl-D-galactosamine	-	-	-
8	*N*-Acetyl-D-glucosamine	+	+	+
9	Adonitol	-	-	+
10	L-Arabinose	+	+	-
11	D-Arabitol	-	-	+
12	D-Cellobiose	-	-	+
13	i-Erythritol	+	+	-
14	D-Fucose	-	-	+
15	L-Fucose	-	-	+
16	D-Galactose	-	-	+
17	Gentiobiose	-	-	-
18	α-D-Glucose	+	+	-
19	m-Inositol	+	+	-
20	α-D-Lactose	-	-	+
21	Lactulose	-	-	+
22	Maltose	-	-	+
23	D-Mannitol	-	-	+
24	D-Mannose	+	+	-
25	D-Melibiose	+	+	-
26	β-Methyl-D-glucoside	-	-	-
27	D-Psicose	+	+	-
28	D-Raffinose	-	-	+
29	L-Rhamnose	-	-	-
30	D-Sorbitol	-	-	-
31	Sucrose	-	-	-
32	D-Trehalose	+	+	-
33	Turanose	-	-	+
34	Xylitol	-	-	+
35	Pyruvic acid methyl ester	+	+	-
36	Succinic acid monomethyl ester	+	+	-
37	Acetic acid	+	+	+
38	*cis*-Aconitic acid	-	-	-
39	Citric acid	-	-	-
40	Formic acid	-	-	-
41	D-Galactonic acid lactone	+	+	-
42	D-Galacturonic acid	-	-	+
43	D-Gluconic acid	+	+	+
44	D-Glucosamic acid	-	-	-
45	D-Glucuronic acid	-	-	-

Table 1. Continued.

No.	Carbon sources for bacterial growth	Xn[1]	Xnk1[2]	Xnk2[3]
46	α-Hydroxybutyric acid	-	-	+
47	β-Hydroxybutyric acid	-	-	+
48	γ-Hydroxybutyric acid	-	-	+
49	p-Hydroxyphenlyacetic acid	+	+	-
50	Itaconic acid	-	-	+
51	α-Ketobutyric acid	-	-	+
52	α-Ketoglutaric acid	-	-	+
53	α-Ketovaleric acid	+	+	+
54	D,L-Lactic acid	+	+	+
55	Malonic acid	-	-	-
56	Propionic acid	+	+	+
57	Quinic acid	-	-	+
58	D-Saccharic acid	-	-	+
59	Sebacic acid	-	-	-
60	Succinic acid	+	+	+
61	Bromosuccinic acid	+	+	+
62	Succinamic acid	-	-	-
63	Glucuronamide	-	-	-
64	L-Alaninamide	+	+	-
65	D-Alanine	+	+	+
66	L-Alanine	+	+	+
67	L-Alanyl-glycine	+	+	-
68	L-Asparagine	+	+	+
69	L-Aspartic acid	+	+	+
70	L-Glutamic acid	+	+	+
71	Glycyl-L-aspartic acid	+	+	-
72	Glycyl-L-glutamic acid	+	+	-
73	L-Histidine	+	+	-
74	Hydroxy-L-proline	-	-	-
75	L-Leucine	-	-	-
76	L-Ornithine	-	-	-
77	L-Phenylalanine	-	-	-
78	L-Proline	+	+	+
79	L-Pyroglutamic acid	-	-	-
80	D-Serine	+	+	+
81	L-Serine	+	+	+
82	L-Threonine	-	-	+
83	D,L-Carnitine	-	-	+
84	γ-Aminobutyric acid	-	-	-
85	Urocanic acid	+	+	+
86	Inosine	+	+	+
87	Uridine	+	+	+
88	Thymidine	+	+	-
89	Phenylethylamine	+	+	-
90	Putrescine	+	+	-
91	2-Aminoethanol	-	-	-
92	2,3-Butanediol	-	-	-
93	Glycerol	+	+	-
94	α-Glycerol phosphate	+	+	+
95	α-D-Glucose-1-phosphate	+	+	+
96	D-Glucose-6-phosphate	+	+	+
	Similarity to Xn	-	99% (96/96)	58% (56/96)

[1]Details of *X. nematophila* are described in *Bergey's Manual* [34].

[2]A primary form of *X. nematophila* isolated from the host nematode, *Steinernema carpocapsae*.

[3]A secondary form of *X. nematophila* after successive *in vitro* cultures.

The Biolog microbial identification system was used to monitor the carbon usage.

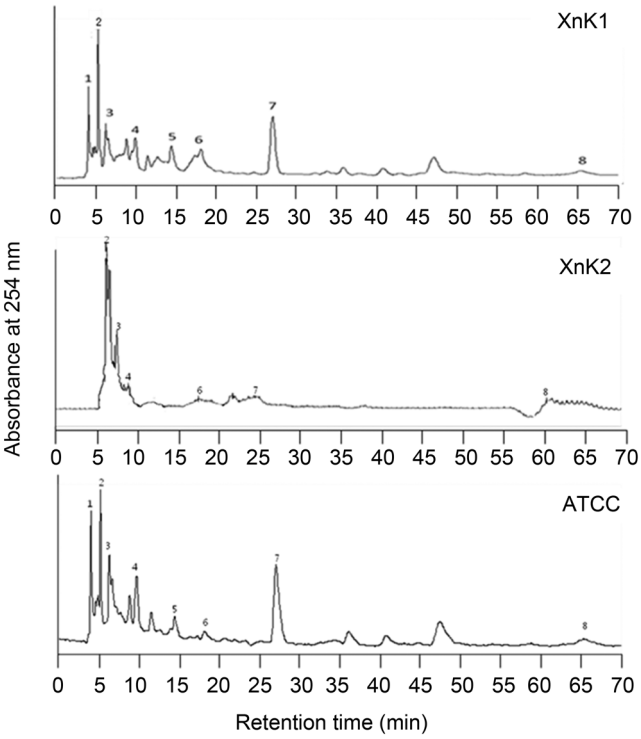

Fig. 1. Chromatograms of three different culture broths of *Xenorhabdus nematophila* (Xn).

"Xn1" and "Xn2" represent primary and secondary forms, respectively. "ATCC" represents an isolate of *X. nematophila* obtained from the American Type Culture Collection (Manassas, VA, USA). The bacteria (5×10^3 CFU) were inoculated to 1 L of TSB culture media and cultured at 28°C for 48 h. Hexane extracts of the culture broth were separated with a reverse-phase C18 HPLC column and analyzed with a UV detector as described in Materials and Methods. Peaks are (1) PHPP, (2) PY, (3) Ac-FGV, (4) cPY, (5) oxindole, (6) HPA, (7) indole, and (8) BZA.

Table 2. Comparison of eight bacterial metabolites produced from three different variants of *X. nematophila*.

Xn strains[1]	Secondary metabolite (mg/ml)[2]							
	Ac-FGV	BZA	Cis-cPY	HPA	Oxindole	indole	PHPP	PY
Xnk1	55.2 ± 11.7a	88.6 ± 10.4a	97.3 ± 21.0a	63.3 ± 19.8a	155.1 ± 49.3a	46.5 ± 18.5a	170.2 ± 61.2a	216.7 ± 50.7a
Xnk2	40.7 ± 23.9b	49.3 ± 2.1b	49.5 ± 5.6b	30.8 ± 8.3b	0 ± 0b	31.2 ± 24.5b	0 ± 0b	63.5 ± 13.0b
ATCC	70.0 ± 19.9a	56.8 ± 3.0a	108.4 ± 37.7a	44.9 ± 12.3a	148.3 ± 64.7a	75.1 ± 33.0a	190.8 ± 132.3a	133.6 ± 83.2a

[1]"Xnk1" is a primary form of *X. nematophila* isolated from the host nematode, *Steinernema carpocapsae*. "Xnk2" is a secondary form of *X. nematophila* after successive *in vitro* cultures. "ATCC" is a primary form of *X. nematophila* obtained from the American Type Culture Collection.

[2]Different letters following standard deviations indicate significance among means at Type I error = 0.05 (LSD test).

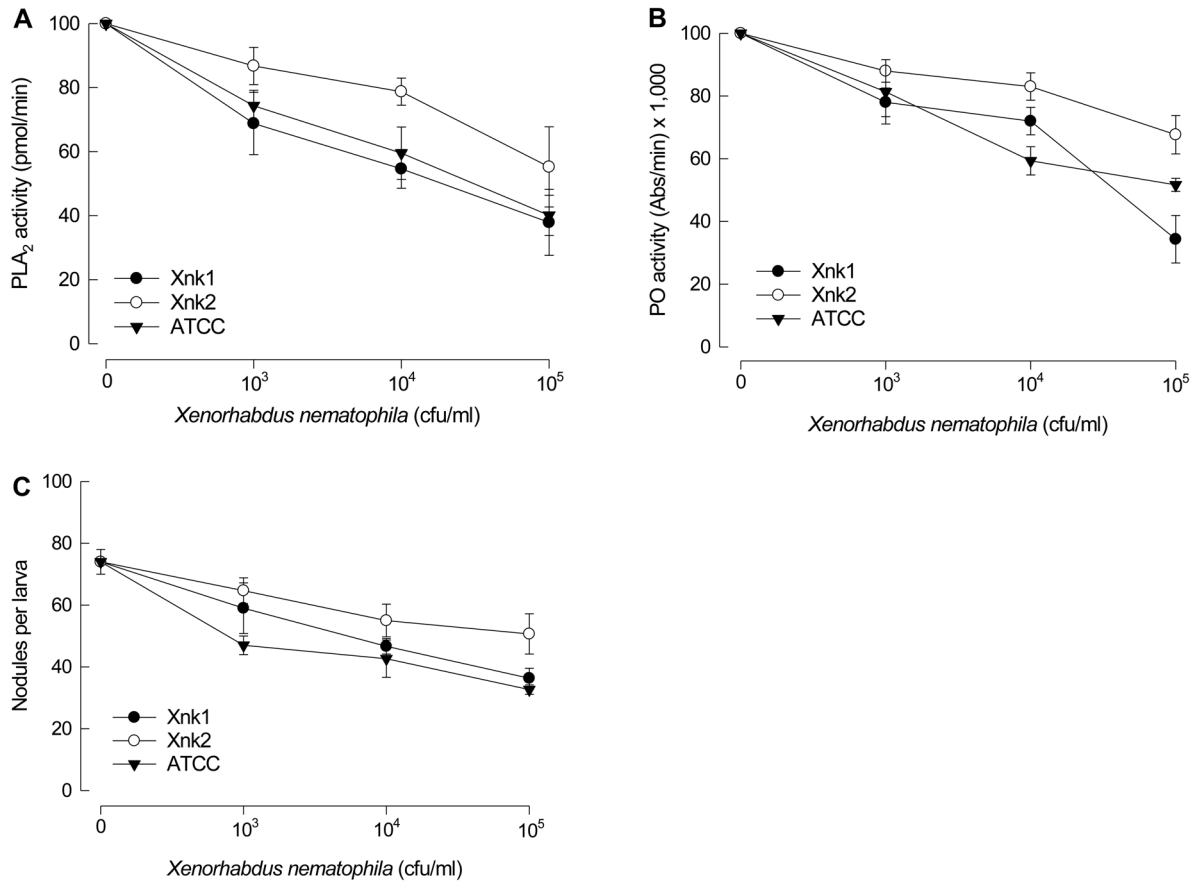

Fig. 2. Reduced activity of the secondary form (Xn2) of *Xenorhabdus nematophila* on three immune-associated responses of *Spodoptera exigua*, compared with those of two primary forms (Xn1 and ATCC).

(**A**) For the PLA_2 enzyme activity assay, fifth instar larvae of *S. exigua* were injected with different numbers of bacteria. After 20 min, the hemolymph was collected and analyzed for enzyme activity using pyrene-labeled phospholipid. Each treatment was replicated three times. (**B**) For the phenoloxidase (PO) activity assay, fifth instar larvae were injected with different numbers of bacteria. After 15 min, the hemolymph was collected and assessed in the PO activity using DOPA as a substrate. Each treatment was replicated three times. (**C**) For the nodulation assay, different numbers of *X. nematophila* were injected to the larvae along with *Escherichia coli* (2×10^4 cells/larva). After 8 h at 25°C, the nodules were counted in the hemocoel. Control represents PBS injection with *E. coli*. Each measurement used five larvae.

form of *X. nematophila* suggested its reduced activities against inhibition of the immunity-associated enzymes or a cellular immunity. First, the PLA_2 activity of the infected larvae was analyzed in the fat body and hemocytes

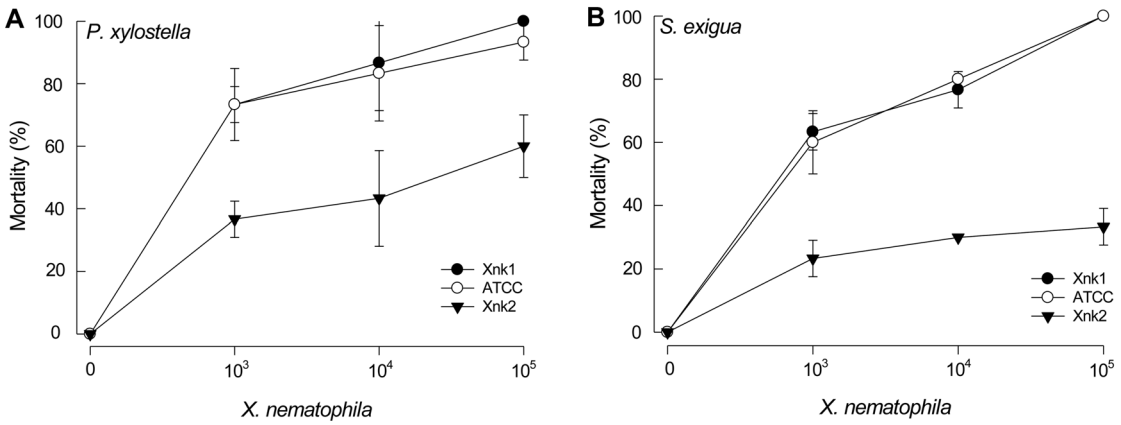

Fig. 3. Reduced pathogenicity of the secondary form (Xn2) of *Xenorhabdus nematophila* on the larvae of *Spodoptera exigua*, compared with those of the two primary forms (Xn1 and ATCC).

Different bacterial numbers were injected to fourth instar *Plutella xylostella* (**A**) or fifth instar *Spodoptera exigua* (**B**) larvae. After 24 h at 25°C, the mortality was assessed. Each treatment was replicated three times. Each replication used 10 larvae.

(Fig. 2A). The larvae infected with Xnk2 showed significantly higher PLA_2 activity ($F = 15.39$; df = 2, 24; $p = 0.0001$), compared with larvae infected with Xnk1 or ATCC strains. Second, PO activity in the hemolymph was significantly higher in larvae infected with Xnk2 than those infected with Xnk1 or ATCC (Fig. 2B). Third, a significantly greater number of hemocyte nodules ($F = 21.1$; df = 2, 24; $p = 0.0001$) was formed in the larvae treated with Xnk2 than those of Xnk1 or ATCC (Fig. 2C). This indicated that XnK2 did not inhibit insect immunity as much as the two primary forms did.

The pathogenic activities of the different *X. nematophila* strains were analyzed by hemocoelic injection (Fig. 3). In both target insects, Xnk2 was significantly lower in pathogenic activity ($F = 19.32$; df = 2, 24; $p = 0.0001$) than Xnk1 or ATCC strains in all treated doses in *P. xylostella* (Fig. 3A) and *S. exigua* (Fig. 3B). Moreover, the rate of lethal effect of Xnk2 progressed much slowly compared with those of both primary forms in the same infection dose (10^3 CFU dose).

Primary Form of *X. nematophila* Significantly Increases Bt Pathogenicity

The culture broth of *X. nematophila* contains the immunosuppressive metabolites, which enhance Bt pathogenicity [53]. A recent production of a commercial Bt-Plus (Lepkill) containing both *X. nematophila* and Bt suffered a low control efficacy, presumably due to a low synergistic effect of *X. nematophila* on Bt pathogenicity. All current data suggested that the low efficacy might be the occurrence of a phase variant of *X. nematophila*. Clearly, use of the primary form of *X. nematophila* significantly enhanced Bt efficacy (Fig. 4). The enhanced efficacy was specific to target insects depending on the Bt strain (Fig. 4A). *B. thuringiensis* subsp. *aizawai* (Bta) was highly effective only against *S. exigua* when it was mixed with Xnk1 (Fig. 4B). On the other hand, *B. thuringiensis* subsp. *kurstaki* (Btk) was highly effective only against *P. xylostella* when it was mixed with Xnk1. When both Bt strains (1:1 = Bta: Btk (v/v)) were combined with the culture broth of Xnk1, the mixture was much more effective against both insect species, but much weaker in its efficacy than the single Bt-containing Bt-Plus (Fig. 4C). For example, in *P. xylostella*, the Bt mixture Bt-Plus (XnK1+Btk+Bta) was more potent than XnK1+Bta, but not as much as XnK1+Btk. Likewise, in *S. exigua*, the Bt mixture Bt-Plus (XnK1+Btk+Bta) was more potent than XnK1+Btk, but not as much as XnK1+Bta. This raised a further analysis to determine the optimal mixture ratio of both Bt strains. A screening test to determine the Bt ratio in Bt-Plus was conducted (Fig. 5). The most effective mixture was a 4:1 ratio of Bta and Btk to effectively control both lepidopteran species. We called the Bt-Plus using Bt mixture as "Dual Bt-Plus".

Addition of BZA or Oxindole Significantly Increases the Insecticidal Activity of Bt-Plus

To further increase the insecticidal activity of Bt-Plus, the effects of the additional bacterial metabolites of *X. nematophila* were assessed (Fig. 6). Among eight metabolites, BZA and oxindole were the most effective to increase the insecticidal activities in both lepidopteran species.

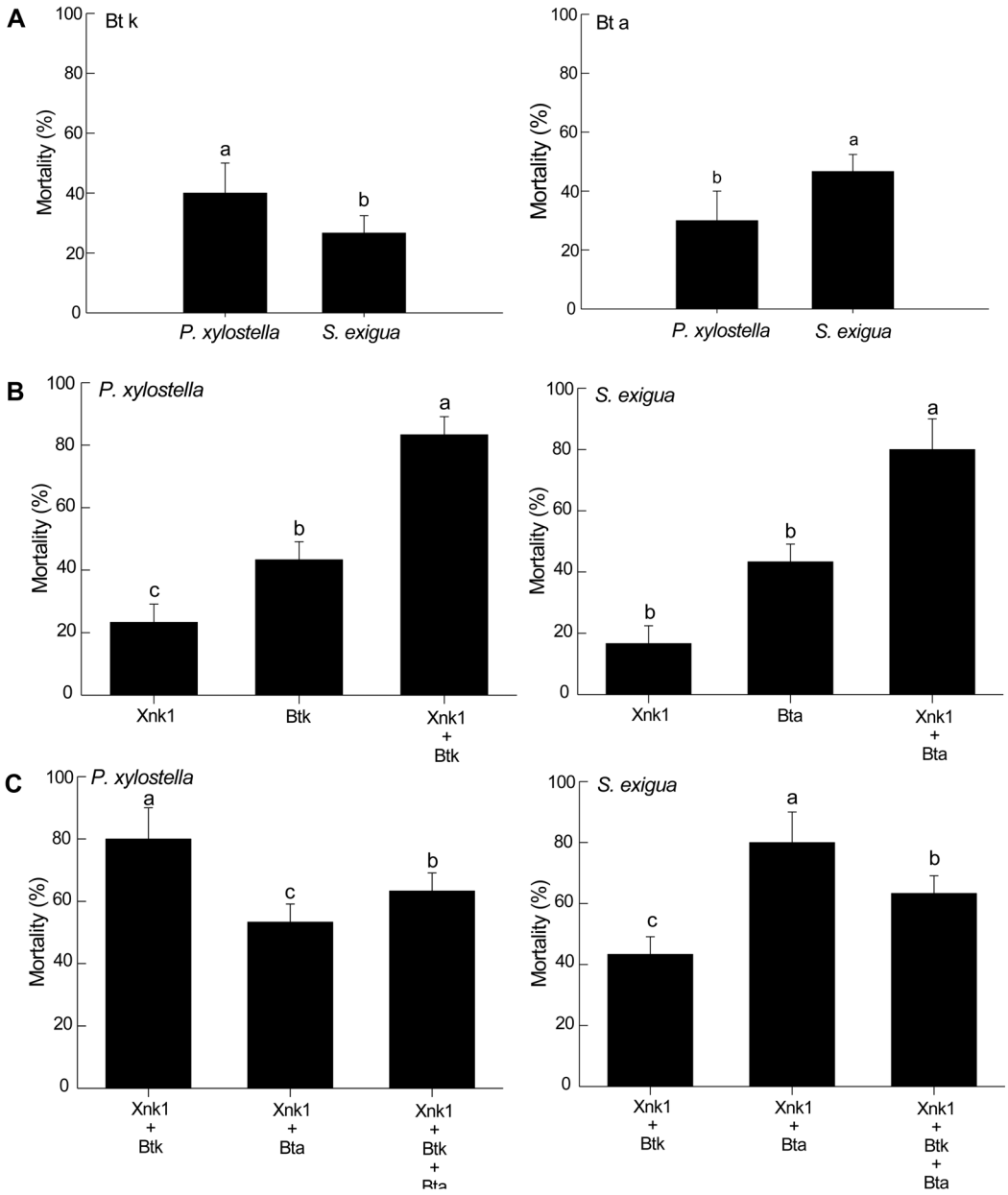

Fig. 4. Enhanced insecticidal effect of Bt-Plus using the primary form of *Xenorhabdus nematophila*.
Two different Bt strains, *B. thuringiensis* ssp. *kurstaki* (Btk) and *B. thuringiensis* ssp. *aizawai* (Bta), were assessed against fourth instar *Plutella xylostella* or fifth instar *Spodoptera exigua* larvae. Each treatment was replicated three times, at which each replication used 10 larvae. Mortality was assessed at 7 days after treatment. (**A**) Insecticidal activities of Bt alone. (**B**) Synergistic effect of *X. nematophila* on Bt toxicity. Bt-Plus (XnK1+Btk or XnK1+Bta) was prepared by mixing two bacterial culture broths in 1:1 ratio. (**C**) Insecticidal activity of a Bt mixture in Bt-Plus (XnK1+Btk+Bta) on both insect species. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test).

These two metabolites were then individually mixed with the Bt-Plus (Fig. 7). Both metabolites significantly increased the insecticidal activities of the Bt-Plus against both *S. exigua* and *P. xylostella* larvae. To confirm the enhanced insecticidal activity of the Dual Bt-Plus fortified with BZA or oxindole, a pot assay was conducted (Table 3). A complete control efficacy was obtained in both insect pests within 4 days.

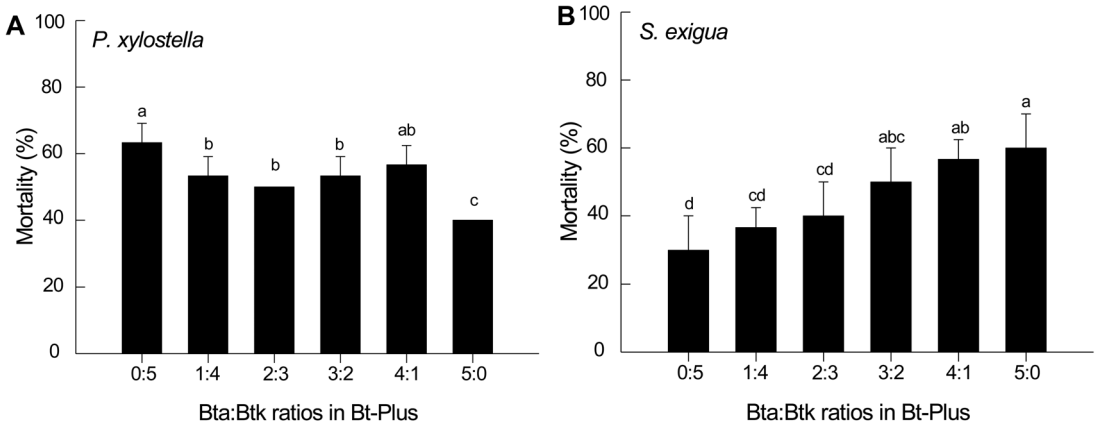

Fig. 5. Determination of an optimal mixture ratio of Bt mixture in Bt-Plus to control both lepidopteran species of fourth instar *Plutella xylostella* and fifth instar *Spodoptera exigua* larvae.

Culture broths of *B. thuringiensis* ssp. *kurstaki* (Btk) and *B. thuringiensis* ssp. *aizawai* (Bta) were mixed in different ratios and added to the culture broth of *Xenorhabdus nematophila* (Xn). A total Bt-Plus consisted of Bt:Xn:ethanol = 5:4:1 (v/v). Each treatment was replicated three times, at which each replication used 10 larvae. Mortality was assessed at 7 days after treatment. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test).

Dual Bt-Plus Using the Concentrated Culture Broth of *X. nematophila*

The fact that the addition of BZA or oxindole significantly increased the insecticidal activity of the Dual Bt-Plus suggested that the concentrated culture broth of *X. nematophila* might be effective because it contained the bacterial metabolites. To test this hypothesis, the culture broth of *X. nematophila* was concentrated 100-fold and used to prepare the Dual Bt-Plus (Fig. 8). Like the metabolite-fortified Bt-Plus (Bt-Plus+oxindole or Bt-Plus+BZA), Dual Bt-Plus using the concentrated culture broth of *X. nematophila* significantly increased the insecticidal activity compared with Bt-Plus. Analysis of bacterial metabolites supported the increases of both BZA and oxindole contents in the Dual Bt-Plus along

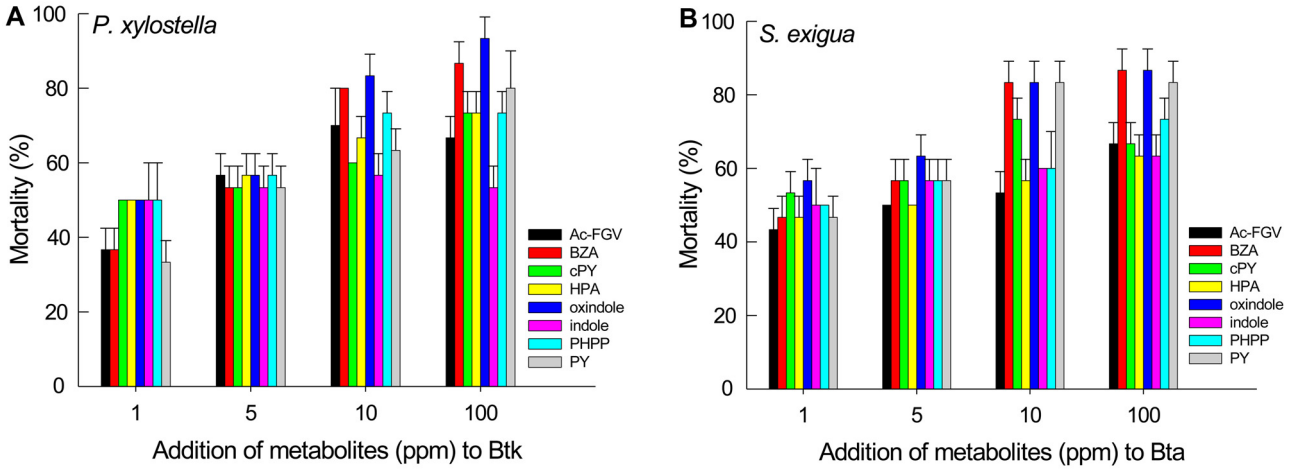

Fig. 6. Effects of the additional bacterial metabolites on Bt toxicity to control fourth instar *Plutella xylostella* or fifth instar *Spodoptera exigua* larvae.

Two different Bt strains, *B. thuringiensis* ssp. *kurstaki* (Btk) and *B. thuringiensis* ssp. *aizawai* (Bta), were used to test the bacterial metabolites in *P. xylostella* and *S. exigua*, respectively. Each treatment was replicated three times, at which each replication used 10 larvae. Mortality was assessed at 7 days after treatment.

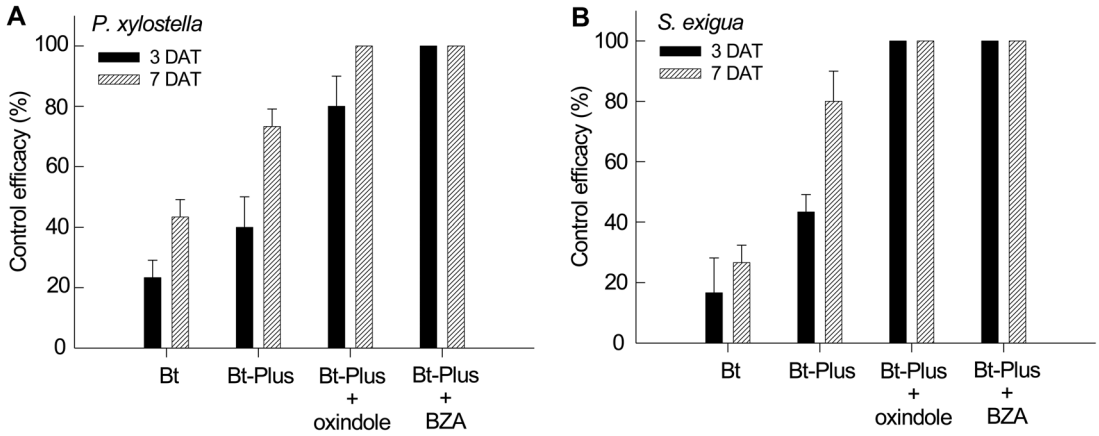

Fig. 7. Effects of two bacterial metabolites on Bt-Plus toxicity to control fourth instar *Plutella xylostella* or fifth instar *Spodoptera exigua* larvae.

Culture broths of *B. thuringiensis* ssp. *kurstaki* (Btk) and *B. thuringiensis* ssp. *aizawai* (Bta) were mixed at 1:4 (Bta:Btk (v/v)) and added to the culture broth of *Xenorhabdus nematophila* (Xn). A total Bt-Plus consisted of Bt:Xn:ethanol = 5:4:1 (v/v). Each 100 ppm of oxindole or BZA was added to the spray suspension of the Bt-Plus in Bt-Plus+oxindole or Bt-Plus+BZA. Each treatment was replicated three times, at which each replication used 10 larvae. Mortality was assessed at 3 and 7 days after treatment (DAT).

with other metabolites, in which most metabolites were detected at 5–10 times increase in Dual Bt-Plus compared with those of Bt-Plus (Table 4). The far less amounts of the bacterial metabolites in the Dual Bt-Plus, even though it used 100-concentrated *X. nematophila*, might be the solubility limits of the nonpolar bacterial metabolites in the spraying suspension.

Discussion

The significance of this study was to develop a novel biopesticide using Bt-Plus containing Bt and *X. nematophila* metabolites to simultaneously control two different lepidopteran insect pests *P. xylostella* and *S. exigua*, which infest the common crop plants in field. To understand the

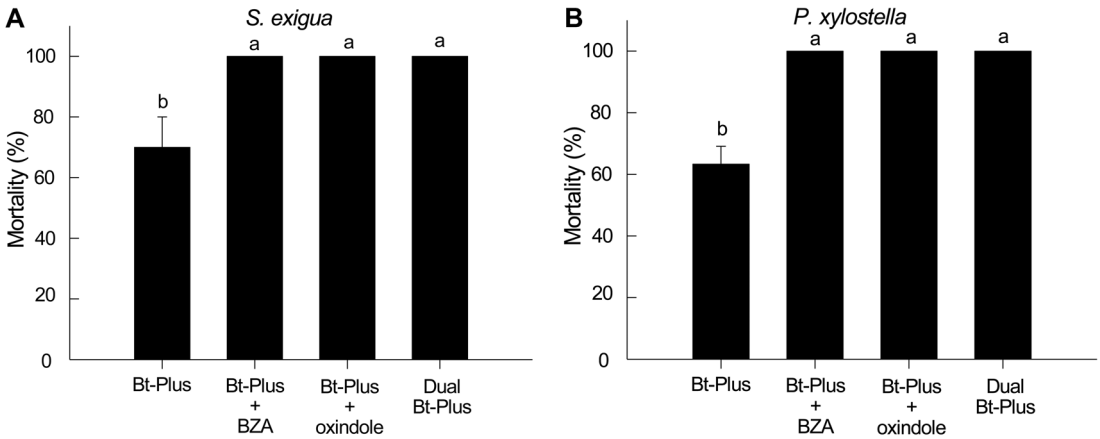

Fig. 8. Development of Dual Bt-Plus by mixing different bacterial culture broths to control fourth instar *Plutella xylostella* or fifth instar *Spodoptera exigua* larvae.

Culture broths of *B. thuringiensis* ssp. *kurstaki* (Btk) and *B. thuringiensis* ssp. *aizawai* (Bta) were mixed and added to the culture broth of *Xenorhabdus nematophila* (Xn). Bt-Plus consisted of a mixture of Bta:Btk:Xn:ethanol = 4:1:4:1 (v/v). Bt-Plus+oxindole or Bt-Plus+BZA was prepared by adding 100 ppm metabolite to the spray suspension of the Bt-Plus. Dual Bt-Plus consisted of a mixture of Bta:Btk:Xn:ethanol = 4:1:4:1 (v/v), in which Xn culture broth was 100-fold concentrated. Each treatment was replicated three times, at which each replication used 10 larvae. Mortality was assessed at 7 days after treatment. Different letters above standard deviation bars indicate significant difference among means at Type I error = 0.05 (LSD test).

Table 3. Control efficacy of Bt-Plus supplemented with the bacterial metabolites of benzylideneacetone (BZA) or oxindole against larvae of (upper) *P. xylostella* and (lower) *S. exigua* in pot assay.

Treatment	N^1	Alive larvae (%) per replication				DMRT	Control efficacy (%)
		1	2	3	Mean		
			3 DAT				
Bt-Plus	46	50.0	63.9	65.2	59.7	b	40.3
Bt-Plus + BZA	42	0.0	0.0	14.3	4.8	a	95.2
Bt-Plus + oxindole	41	12.2	19.5	29.3	20.3	ab	79.7
Untreated	41	96.3	99.1	94.3	96.6	c	-
			7 DAT				
Bt-Plus	46	16.8	34	33.2	28	b	72.0
Bt-Plus + BZA	42	0.0	0.0	0.0	0.0	a	100
Bt-Plus + oxindole	41	12.2	19.5	0.0	0.0	a	100
Untreated	41	96.3	99.1	94.3	96.6	c	-

Treatment	N^1	Alive larvae (%) per replication				DMRT	Control efficacy
		1	2	3	Mean		
			3 DAT				
Bt-Plus	46	54.4	61.8	59.2	58.5	b	41.5
Bt-Plus + BZA	42	0.0	0.0	0.0	0.0	a	100
Bt-Plus + oxindole	44	0.0	0.0	0.0	0.0	a	100
Control	43	98.3	100	96.3	98.2	c	-
			7 DAT				
Bt-Plus	46	11.2	26.4	30.5	22.7	b	77.3
Bt-Plus + BZA	42	0.0	0.0	0.0	0.0	a	100
Bt-Plus + oxindole	44	0.0	0.0	0.0	0.0	a	100
Control	43	98.3	100	96.3	98.2	c	-

[1]Average initial density per replication.

Three cabbage plants in a pot were infested with late instar larvae. Each treatment was replicated with three pots. Mortality was assessed at 3 and 7 days after treatment (DAT). Different letters represent significant difference in mean survivals of different treatments at Type I error = 0.05 (Multiple mean range test: DMRT).

Bt-Plus efficacy and its development to control both insect targets, Bt pathogenicity and resistance mechanisms need to be described. Bt has been widely used as a biopesticide to control lepidopteran, coleopteran, and dipteran insect pests. Bt Cry toxins are used to breed genetically modified crops, such as Bt-corn, Bt-soybean, and Bt-cotton, which drastically increase in cultivating areas over the world [64]. As like other insecticides, target insects develop resistance against various Bt strains in the field as well as in laboratories [20]. The major resistance mechanism is derived

Table 4. Quantitative analysis of eight bacterial metabolites in different Bt-Plus products.

Treatment	Bacterial metabolite (μg/ml)[1]							
	Ac-FGV	BZA	cPY	HPA	Indole	Oxindole	PHPP	PY
Bt-Plus	28.3 ± 5.0b	30.7 ± 8.4c	51.6 ± 6.0b	14.3 ± 5.0b	11.9 ± 2.3b	79.1 ± 12.8c	88.5 ± 23.7b	90.9 ± 17.2b
Bt-Plus + BZA	31.7 ± 5.0b	94.1 ± 12.0b	53.3 ± 1.0b	9.4 ± 0.8b	30.7 ± 2.0b	69.4 ± 4.0c	82.6 ± 9.0b	74.3 ± 3.0b
Bt-Plus + Oxindole	28.1 ± 4.0b	18.7 ± 6.0c	38.0 ± 7.0b	11.3 ± 1.0b	28.4 ± 8.0b	246.3 ± 18.0b	90.2 ± 6.0b	77.5 ± 6.0b
Dual Bt-Plus	160.1 ± 44.7a	187.9 ± 27.4a	456.2 ± 63.6a	96.8 ± 11.3a	130.3 ± 31.1a	613.7 ± 89.6a	785.4 ± 149.8a	813.0 ± 105.8a

[1]Different letters following standard deviations indicate significance among means at Type I error = 0.05 (LSD test).

from the interaction between Bt Cry toxins and insect midgut epithelium [46]. When Bt spores are consumed, they germinate and the crystals associated with spores are dissolved in the alkaline juice in the midgut lumen. The protoxin is then cleaved by insect digestive proteases to produce the active, protease-insensitive toxin core protein. The active toxins migrate to the ectoperitrophic space through the peritrophic membrane and reversibly or irreversibly interact with molecules in the epithelial membrane. Aggregation of toxins into oligomers happens and disrupts the epithelial membrane by pore formation, which results in cell lysis called "colloid-osmotic lysis" [32]. The damaged midgut epithelial membranes cause a complete cessation of feeding behavior or a fatal proliferation of Bt and other microorganisms to kill insects [8].

Bt resistance can be induced by interruption of any of the steps of Bt pathogenicity described above. A Bt resistance may be derived from interruption of toxin activation from the inactive para-crystal form. This resistance mechanism includes overexpression of proteases to degrade the protoxins [56], sequestering the toxins by precipitation [41] or coagulation [40] or trapping in the peritrophic matrix. Modification of the Bt toxin-binding sites in the epithelial membrane reduces or prevents the irreversible binding, which is crucial to Bt toxicity [13, 65]. Furthermore, pore formation can be interfered with or pores can be plugged [55]. Dead midgut cells can be replaced by the increase of stem cell proliferation [38]. Finally, the elevated immune response can effectively defend Bt and other enteric bacterial infection to the hemocoel [48]. The role of the elevated immune responses has been demonstrated by increase of melanization immune response in *Ephestia kuehniella* and *Helicoverpa armigera* that exhibited 16-fold and 12-fold greater tolerances to Cry toxins [40, 48]. In *S. exigua*, the Bt-resistant strain elevated the gene expression associated with immune responses [21]. The significant increase of Bt toxicity in Bt-Plus can be explained by the immunosuppression induced by PLA_2-inhibitory metabolites of *X. nematophila*. All eight metabolites are potent to inhibit PLA_2 activity to prevent the production of eicosanoids, which play crucial roles in mediating insect immunity [63]. Furthermore, these eight metabolites are effective to inhibit PO activity, which is crucial to form coagulation of Bt Cry toxins in the midgut lumen [49]. Thus, the metabolites of *X. nematophila* in Bt-Plus suppress target insect immunity by inhibiting PLA_2 and PO to facilitate Bt toxin activation and induce the fatal septicemia in the target insect hemocoel.

A phase variation is well documented in *X. nematophila* [14, 59]. Initially, Akhurst [1] demonstrated that *X. nematophila* produces two colony types on agar media. The primary (phase I) form, isolated from the nematode intestine, is unstable *in vitro* and produces the secondary (phase II) form, which is well adapted to grow *in vitro* [5]. The common characters of the primary form include motility, dye absorption, antibiotics production, hemagglutination, and synthesis of the outer membrane protein, OpnB [16, 17, 67]. However, the secondary form characters of *X. nematophila* are variable depending on strains [7]. Our secondary form was distinct in bromophenol blue dye absorption, insect pathogenicity, and the production of the secondary metabolites, compared with those of the primary form isolated from the nematode *S. carpocapsae*. The decreased pathogenicity of the secondary form is partially explained by the decrease in the production of the bacterial metabolites, which are potent to inhibit insect immune responses [54]. However, both primary and secondary forms of *X. nematophila* are equally pathogenic to *Galleria mellonella* [10] and functional in the *in vitro* culture of host nematodes [12]. On the other hand, the decrease of insecticidal activity is observed from a secondary form of *X. nematophila* possessing a Lrp (leucine-responsive regulatory protein) mutant in *Manduca sexta* [9]. The variation of the secondary forms of *X. nematophila* may be explained in its origin of a random mutation of a global regulator [50, 67]. Thus, the percent decrease of Bt-Plus efficacy must be the occurrence of the secondary form of *X. nematophila*.

With the primary form of *X. nematophila*, a Dual Bt-Plus was developed to control both *P. xylostella* and *S. exigua*. Usually, *P. xylostella* becomes more susceptible to CryIA (a main Cry toxin of Btk) than Cry1C (a main Cry toxin of Bta), whereas *S. exigua* becomes more susceptible to Cry1C than Cry1A [39, 68]. In this current study, an optimal ratio of Bta and Btk (4:1 in spore density) was relatively effective to control both insect species. With respect to Bt toxicity, active Cry toxins are concentrated on the midgut membrane receptors, such as alkaline phosphatase (ALP) or aminopeptidase N (APN), and interact with cadherin molecules on the membrane for further cleavage at the N-terminal piece [2, 42]. The cleaved Cry toxins then bind to each other to form an oligomeric prepore structure, which exhibits high binding activity to both ALP and APN [19, 60]. Then ALP and APN facilitate the pore formation of the oligomers on the midgut membrane. The oligomerization of the Cry toxins has been regarded as a crucial step for the toxicity of Bt Cry toxins [43]. Thus, our results of the optimal Bt mixture exhibiting high toxicity to both lepidopteran species suggests a hetero-oligomerization of different Cry toxins, which may further facilitate a cooperative oligomerization

between different Cry toxins to form pores in the epithelial membrane of the midgut. In fact, the mixture of Cry1Ab and Cry1Ac exhibited 5-fold synergistic toxicity against *Chilo partellus* larvae [57]. Similarly, Cry1Aa and Cry1Ac have a synergistic effect against *Lymantria dispar* larvae, increasing their toxicity by 4.9-fold when the larvae were fed with a mixture of toxins, in which the combination of the two toxins resulted in greater pore formation activity than the individual toxins [36]. Most Bt strains produce more than one type of Cry toxins, suggesting that hetero-oligomerization of different Cry toxins may be favored during evolution of Cry toxins as a mechanism to exploit the target insects for their specific and cooperative toxicity through an optimal pore formation strategy.

To further increase the Bt-Plus efficacy to both insect species, the specific bacterial metabolites were added after selecting the most potent metabolites (BZA and oxindole). The two metabolites possess PLA_2/PO inhibition and cytotoxicity [54]. Furthermore, BZA also exhibits an independent oral toxicity by suppressing the digestive efficiency of *S. exigua* [29]. In addition, the dual Bt-Plus fortified with BZA or oxindole exhibited a complete control efficacy in a pot assay. This suggests that the 100-fold concentrated culture broth of *X. nematophila* can be an alternative choice to prepare a dual Bt-Plus without adding BZA or oxindole because of the presence of ≈1 ppm of these compounds in the current Bt-Plus formulation. Indeed, the 100-fold concentrated culture broth of *X. nematophila* effectively increased the Bt-Plus insecticidal activity like the metabolite-fortified Bt-Plus.

These results recapitulate the crucial role of the primary form of *X. nematophila* to enhance insecticidal activity. With the primary form of *X. nematophila*, this study developed a novel biopesticide to effectively control both *S. exigua* and *P. xylostella* by combining both types of Cry toxins with the concentrated culture broth of *X. nematophila* to meet the effective concentration of BZA or oxindole.

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References

1. Akhurst RJ. 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *J. Gen. Microbiol*. **121:** 303-309.
2. Arenas I, Bravo A, Soberón M, Gómez I. 2010. Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. *J. Biol. Chem*. **285:** 12497-12503.
3. Beckage NE. 2010. *Insect Immunology*. Academic Press, NY.
4. BenFarhat D, Dammak M, Khedher SB, Mahfoudh S, Kammoun S, Tounsi S. 2013. Response of larval *Ephestia kuehniella* (Lepidoptera: Pyralidae) to individual *Bacillus thuringiensis kurstaki* mixed with *Xenorhabdus nematophila*. *J. Invertebr. Pathol*. **114:** 71-75.
5. Boemare NE, Akhurst RJ. 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp. (Enterobacteriaceae). *J. Gen. Microbiol*. **134:** 751-761.
6. Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **71:** 248-254.
7. Brehelin MA, Cherqui L, Drif L, Luciani L, Akhurst RJ, Boemare NE. 1993. Ultrastructure study of surface components of *Xenorhabdus* sp. in different cell phase and culture conditions. *J. Invertebr. Pathol*. **61:** 188-191.
8. Broderick NA, Raffa KF, Handelsman J. 2006. Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. *Proc. Natl. Acad. Sci. USA* **103:** 15196-15199.
9. Cowles KN, Cowles CE, Richards GR, Martens EC, Goodrich-Blair H. 2007. The global regulator Lrp contributes to mutualism, pathogenesis and phenotypic variation in the bacterium *Xenorhabdus nematophila*. *Cell. Microbiol*. **9:** 1311-1323.
10. Dunphy GB, Webster JM. 1988. Interaction of *Xenorhabdus nematophilus* subsp. *nematophilus* with the haemolymph of *Galleria mellonella*. *Int. J. Parasitol*. **30:** 883-889.
11. Dunphy GB, Webster JM. 1991. Antihemocytic surface components of *Xenorhabdus nematophilus* var. *dutki* and their modification by serum of nonimmune larvae of *Galleria mellonella*. *J. Invertebr. Pathol*. **58:** 40-51.
12. Ehlers RU, Stoessel S, Wyss U. 1990. The influence of phase variants of *Xenorhabdus* spp. and *Escherichia coli* (Enterobacteriaceae) on the propagation of entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*. *Rev. Nematol*. **13:** 417-424.
13. Ferré J, Real MD, van Rie J, Jansens S, Peferoen M. 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. *Proc. Natl. Acad. Sci. USA* **88:** 5119-5123.
14. Forst S, Clarke D. 2002. Bacteria-nematode symbioses, pp. 57-77. *In* Gaugler R (ed.). *Entomopathogenic Nematology*. CABI Publishing, Wallingford.
15. Forst S, Dowds B, Boemare N, Stackebrandt E. 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs.

Annu. Rev. Microbiol. **51:** 47-72.
16. Forst S, Tabatabai N. 1997. Role of the histidine kinase, EnvZ, in the production of outer membrane proteins in the symbiotic-pathogenic bacterium, *Xenorhabdus nematophilus*. *Appl. Environ. Microbiol*. **63:** 962-968.
17. Givaudan A, Baghdiguian S, Lanois A, Boemare N. 1995. Swarming and swimming changes concomitant with phase variation in *Xenorhabdus nematophilus*. *Appl. Environ. Microbiol*. **61:** 1408-1413.
18. Goh HG, Lee SG, Lee BP, Choi KM, Kim JH. 1990. Simple mass-rearing of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), on an artificial diet. *Kor. J. Appl. Entomol*. **29:** 180-183.
19. Gómez I, Sánchez J, Miranda R, Bravo A, Soberón M. 2002. Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Lett*. **513:** 242-246.
20. Heckel DG, Gahan LJ, Baxter SW, Zhao JZ, Shelton AM, Gould F, *et al*. 2007. The diversity of Bt resistance genes in species of Lepidoptera. *J. Invertebr. Pathol*. **95:** 192-197.
21. Hernandez-Martinez P, Navarro-Cerrillo G, Caccia S, de Maagd RA, Moar WJ, Ferré J, *et al*. 2010. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. *PLoS ONE* **17:** e12795.
22. Haine ER, Moret Y, Siva-Jothy MT, Rolff J. 2008. Antimicrobial defense and persistent infection in insects. *Science* **322:** 1257-1259.
23. Humason GL. 1972. *Animal Tissue Techniques*, 3rd Ed. W.H. Freeman and Company, San Francisco, CA.
24. Ji D, Yi Y, Kang GH, Choi YH, Kim P, Baek NI, *et al*. 2004. Identification of an antibacterial compound, benzylideneacetone, from *Xenorhabdus nematophila* against major plant-pathogenic bacteria. *FEMS Microbiol. Lett*. **239:** 241-248.
25. Ji D, Yi Y, Kim Y. 2004. 16S rDNA sequence and biochemical characters of a Korean isolate of *Xenorhabdus nematophila*. *J. Asia Pac. Entomol*. **7:** 105-111.
26. Jung C, Kim Y. 2006. Synergistic effect of entomopathogenic bacteria (*Xenorhabdus* sp. and *Photorhabdus temperata* subsp. *temperata*) on the pathogenicity of *Bacillus thuringiensis* ssp. *aizawai* against *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Entomol*. **35:** 1584-1589.
27. Jung C, Kim Y. 2006. Potentiating effect of *Bacillus thuringiensis* ssp. *kurstaki* on pathogenicity of entomopathogenic bacterium *Xenorhabdus nematophila* K1 against diamondback moth, *Plutella xylostella*. *J. Econ. Entomol*. **100:** 246-250.
28. Kaya HK, Gaugler R. 1993. Entomopathogenic nematodes. *Annu. Rev. Entomol*. **38:** 181-206.
29. Kim J, Kim Y. 2011. Three metabolites from an entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibit larval development of *Spodoptera exigua* (Lepidoptera: Noctuidae) by inhibiting a digestive enzyme, phospholipase A_2. *Insect Sci.* **18:** 282-288.
30. Kim K, Park Y, Kim Y, Lee Y. 2001. Study on the inoculation augmentation of *Paecilomyces japonicus* to the silkworm, *Bombyx mori*, using dexamethasone. *Kor. J. Appl. Entomol*. **40:** 51-58.
31. Kim Y, Ji D, Cho S, Park Y. 2005. Two groups of entomopathogenic bacteria, *Photorhabdus* and *Xenorhabdus*, share an inhibitory action against phospholipase A_2 to induce host immunodepression. *J. Invertebr. Pathol*. **89:** 258-264.
32. Knowles BH, Ellar DJ. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action on *Bacillus thuringiensis* delta-endotoxins with different insect specificity. *Biochim. Biophys. Acta* **924:** 509-518.
33. Koppenhöfer AM, Grewal PS, Fuzy EM. 2007. Differences in penetration routes and establishment rates of four entomopathogenic nematode species into four white grub species. *J. Invertebr. Pathol*. **94:** 184-195.
34. Krieg NR, Hort JG. 1984. *Bergey's Manual of Systematic Bacteriology*, pp. 506-512. Vol. 1. Williams and Wilkins, Baltimore.
35. Lavine MD, Strand MR. 2002. Insect hemocytes and their role in cellular immune responses. *Insect Biochem. Mol. Biol*. **32:** 1237-1242.
36. Lee MK, Curtiss A, Alcantara E, Dean DH. 1996. Synergistic effect of the *Bacillus thuringiensis* toxins Cry1Aa and Cry1Ac on the gypsy moth, *Lymantria dispar*. *Appl. Environ. Microbiol.* **62:** 583-586.
37. Lemaitre B, Hoffmann J. 2007. The host defense of *Drosophila melanogaster*. *Annu. Rev. Immunol.* **25:** 697-743.
38. Loeb MJ, Martin PAW, Hakim RS, Goto S, Takeda M. 2001. Regeneration of cultured midgut cells after exposure to sublethal doses of toxin from two strains of *Bacillus thuringiensis*. *J. Insect Physiol*. **47:** 599-606.
39. Luo K, Banks D, Adang MJ. 1999. Toxicity, binding, and permeability analyses of four *Bacillus thuringiensis* Cry1 δ-endotoxins using brush border membrane vesicles of *Spodoptera exigua* and *Spodoptera frugiperda*. *Appl. Environ. Microbiol*. **65:** 457-464.
40. Ma G, Roberts H, Sarjan M, Featherstone N, Lahnstein J, Akhurst R, *et al*. 2005. Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant, *Helicoverpa armigera* larvae? *Insect Biochem. Mol. Biol*. **35:** 729-739.
41. Milne R, Wright T, Kaplan H, Dean D. 1998. Spruce budworm elastase precipitates *Bacillus thuringiensis* delta-endotoxin by specifically recognizing the C-terminal region. *Insect Biochem. Mol. Biol*. **28:** 1013-1023.
42. Pacheco S, Gómez I, Arenas I, Saab-Rincon G, Rodrigues-Almazan C, Gill SS, *et al.* 2009. Domain II loop 3 of *Bacillus thuringiensis* Cry1Ab toxin is involved in a "ping-pong" binding mechanism with *Manduca sexta* aminopeptidase-N and cadherin receptors. *J. Biol. Chem*. **284:** 32750-32757.
43. Pardo-Lopez L, Soberon M, Bravo A. 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. *FEMS Microbiol*. **37:** 3-22.
44. 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.

45. Park Y, Kim Y. 2003. *Xenorhabdus nematophila* inhibits *p*-bromophenacyl bromide (BPB)-sensitive PLA_2 of *Spodoptera exigua*. *Arch. Insect Biochem. Physiol*. **54:** 134-142.
46. Pietrantonio PV, Gill SS. 1996. *Bacillus thuringiensis* toxins: action on the insect midgut, pp. 345-372. *In* Lehane MJ, Billingsley PE (eds.). *Biology of the Insect Midgut*. Chapman & Hall, London,
47. Radvanyi F, Jordan L, Russo-Marie F, Bon C. 1989. A sensitive and continuous fluorometric assay for phospholipase A_2 using pyrene-labeled phospholipids in the presence of serum albumin. *Anal. Biochem*. **177:** 103-109.
48. Rahman MM, Roberts HLS, Sarjan M, Asgari S, Schmidt O. 2004. Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*. *Proc. Natl. Acad. Sci. USA* **101:** 2696-2699.
49. Rahman MM, Roberts HLS, Schmidt O. 2007. Tolerance to *Bacillus thuringiensis* endotoxin in immune-suppressed larvae of the flour moth *Ephestia kuehniella*. *J. Invertebr. Pathol*. **96:** 125-132.
50. Richards GR, Goodrich-Blair H. 2009. Masters of conquest and pillage: *Xenorhabdus nematophila* global regulators control transitions from virulence to nutrient acquisition. *Cell. Microbiol*. **11:** 1025-1033.
51. SAS Institute, Inc. 1989. *SAS/STAT User's Guide*, release 6.03 Ed. SAS Institute, Cary, NC.
52. Seo S, Kim Y. 2010. Study on development of novel biopesticides using entomopathogenic bacterial culture broth of *Xenorhabdus* and *Photorhabdus*. *Kor. J. Appl. Entomol.* **49:** 241-249.
53. Seo S, Kim Y. 2011. Development of "Bt-Plus" biopesticide using entomopathogenic bacteria (*Xenorhabdus nematophila*, *Photorhabdus temperata* ssp. *temperata*) metabolites. *Kor. J. Appl. Entomol*. **50:** 171-178.
54. Seo S, Lee S, Hong Y, Kim Y. 2012. Phospholipase A_2 inhibitors synthesized by two entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus temperata* subsp. *temperata*. *Appl. Environ. Microbiol*. **78:** 3816-3823.
55. Shai Y. 2001. Molecular recognition within the membrane milieu: implications for the structure and function of membrane proteins. *J. Membr. Biol*. **182:** 91-104.
56. Shao ZZ, Cui YL, Liu XL, Yi HQ, Ji JH, Yu ZN. 1998. Processing of delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. *J. Invertebr. Pathol*. **72:** 73-81.
57. Sharma A, Nain V, Lakhanpaul S, Kumar PA. 2010. Synergistic activity between *Bacillus thuringiensis* Cry1Ab and Cry1Ac toxins against maize stem borer (*Chilo partellus* Swinhoe). *Lett. Appl. Microbiol*. **51:** 42-47.
58. Shrestha S, Kim Y. 2009. Various eicosanoids modulate the cellular and humoral immune responses of the beet armyworm, *Spodoptera exigua*. *Biochem. Biophys. Biotech*. **73:** 2077-2084.
59. Smits WK, Kuipers OP, Veening JW. 2006. Phenotypic variation in bacteria the role of feedback regulation. *Nat. Rev. Microbiol*. **4:** 259-271.
60. Sóberon M, Pardo-López L, López I, Gómez I, Tabashnik B, Bravo A. 2007. Engineering modified Bt toxins to counter insect resistance. *Science* **318:** 1640-1642.
61. Stanley DW. 2000. *Eicosanoids in Invertebrate Signal Transduction Systems*. Princeton University Press, Princeton, NJ.
62. Stanley DW. 2006. Prostaglandins and other eicosanoids in insects: biological significance. *Annu. Rev. Entomol*. **51:** 25-44.
63. Stanley D, Kim Y. 2011. Prostaglandins and their receptors in insect biology. *Front. Endocrinol*. **2:** 1-11.
64. Tabashnik BE, Brévault T, Carrière Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. *Nature Biotech*. **31:** 510-521.
65. van Rie J, McGaughey WH, Johnson DE, Barnett BD, van Mellaert H. 1990. Mechanism of insect resistance to the microbial insecticide, *Bacillus thuringiensis*. *Science* **247:** 72-74.
66. Vigneux F, Zumbihl R, Jubelin G, Ribeiro C, Poncet J, Baghdiguian S, *et al*. 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*. **13:** 9571-9580.
67. Volgyi A, Fodor A, Forst S. 2000. Inactivation of a novel gene produces a phenotypic variant cell and affects the symbiotic behavior of *Xenorhabdus nematophilus*. *Appl. Environ. Microbiol.* **66:** 1622-1628.
68. Wright DJ, Iqbal M, Granero F, Ferre J. 1997. Change in a single midgut receptor in the diamondback moth (*Plutella xylostella*) is only in part responsible for field resistance to *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *aizawai*. *Appl. Environ. Microbiol*. **63:** 1814-1819.