Virulent secondary metabolites of entomopathogenic bacteria genera, *Xenorhabdus* and *Photorhabdus*, inhibit phospholipase A₂ to suppress host insect immunity

Md. Mahi Imam Mollah and Yonggyun Kim*

Abstract

**Background:** *Xenorhabdus* and *Photorhabdus* are entomopathogenic bacteria that cause septicemia and toxemia in insects. They produce secondary metabolites to induce host immunosuppression. Their metabolite compositions vary among bacterial species. Little is known about the relationship between metabolite compositions and the bacterial pathogenicity. The objective of this study was to compare pathogenicity and production of secondary metabolites of 14 bacterial isolates (species or strains) of *Xenorhabdus* and *Photorhabdus*.

**Results:** All bacterial isolates exhibited insecticidal activities after hemocoelic injection to *Spodoptera exigua* (a lepidopteran insect) larvae, with median lethal doses ranging from 168.8 to 641.3 CFU per larva. Bacterial infection also led to immunosuppression by inhibiting eicosanoid biosynthesis. Bacterial culture broth was fractionated into four different organic extracts. All four organic extracts of each bacterial species exhibited insecticidal activities and resulted in immunosuppression. These organic extracts were subjected to GC-MS analysis which predicted 182 compounds, showing differential compositions for 14 bacteria isolates. There were positive correlations between total number of secondary metabolites produced by each bacterial culture broth and its bacterial pathogenicity based on immunosuppression and insecticidal activity. From these correlation results, 70 virulent compounds were selected from secondary metabolites of high virulent bacterial isolates by deducting those of low virulent bacterial isolates. These selected virulent compounds exhibited significant immunosuppressive activities by inhibiting eicosanoid biosynthesis. They also exhibited relatively high insecticidal activities.

**Conclusion:** Virulence variation between *Xenorhabdus* and *Photorhabdus* is determined by their different compositions of secondary metabolites, of which PLA₂ inhibitors play a crucial role.

**Keywords:** *Xenorhabdus*, *Photorhabdus*, Secondary metabolite, Pathogenicity, Eicosanoid, Immunity
Background

Xenorhabdus and Photorhabdus are entomopathogenic bacteria that exhibit mutualistic symbiosis with entomopathogenic nematodes Steinernema and Heterorhabditis, respectively [1]. These nematodes can carry and release these bacteria into insect hemocoel by infecting susceptible insect larvae, in which these bacteria can kill insects and convert the cadaver into a food source suitable for nematode growth and development [2]. When nematode population increases to a certain carrying level in a specific insect host, these nematodes are re-associated with specific symbiotic bacteria before emerging from the insect cadaver to search for a new host [3]. There are complex chemical communications in tripartite interactions of bacteria-nematode for symbiosis, bacteria-insect for pathogenicity, and nematode-insect for host recognition. Pathogenic interactions between bacteria and susceptible insects have been relatively well studied regarding the production of specialized metabolites derived from non-ribosomal peptide synthetase (NRPS) or polyketide synthase (PKS) [4]. However, there are secondary compounds other than NRPS-PKS produced from synthetic machineries of bacteria [5].

Virulence of entomopathogenic bacteria exhibits variations among species and strains [6, 7]. Bacteria can secrete several virulence factors in insect hemocoel to suppress insect immune responses and cause fatal septicemia [8, 9]. To induce immunosuppression, both bacterial genera commonly inhibit phospholipase A2 (PLA2) activity of insects [10]. PLA2 is known to catalyze the release of arachidonic acid from phospholipids, which is a committed step to produce various eicosanoids [11]. Eicosanoids mediate cellular and humoral immune responses against various microbial pathogens in insects [12]. Indeed, X. nematophila can secrete at least eight secondary metabolites to suppress insect immunity by inhibiting PLA2 [13]. Inter-specific variations in Xenorhabdus bacterial virulence have been explained by variations in their inhibitory activities against PLA2 activities [14]. Intra-specific variations in bacterial virulence of X. nematophila have also been reported and explained by differences in immunosuppression due to differential inhibitory effects on PLA2 catalytic activity [15]. Park et al. [16] have explained that different virulence due to phase variation is associated with the expression of a specific outer membrane protein. This discovery on virulence gene is further extended by the finding that the expression level of leucine-responsive protein (Lrp), a global transcriptional factor, can modulate bacterial pathogenicity [17]. Expression levels of Lrp and other transcriptional factors can modulate secondary metabolite production [18], suggesting that the production of secondary metabolites might be positively correlated with bacterial pathogenicity.

Secondary metabolites produced by NRPS and PKS of different species of Xenorhabdus and Photorhabdus are different in their compositions [19]. Different secondary metabolites can inhibit diverse physiological molecules of susceptible insects to induce immunosuppression. For example, rhabdopicin, an isocyanide-containing compound produced from biosynthetic gene cluster, can inhibit the activity of phenoloxidase (PO) in Galleria mellonella [20]. More than 70 kinds of rhabdopeptide/xenortide peptides derived from NRPS are structurally similar to protease inhibitors. They might degrade various proteins associated with immunity [21, 22]. Phurealipids produced from NRPS/PKS can prevent the expression of antimicrobial peptide genes [23]. Thus, diverse secondary metabolites produced by entomopathogenic bacteria might effectively suppress insect immune responses to induce septicemia.

The aim of this study was to determine virulent secondary metabolites produced by Xenorhabdus or Photorhabdus based on their inhibitory activities against insect PLA2. To this end, this study compared virulence and secondary metabolites of 14 different bacterial isolates of X. nematophila and X. hominickii.

Results

Variations in bacterial virulence

Insecticidal activities of 14 bacterial isolates (species or strains) of Xenorhabdus and Photorhabdus were assessed by hemocoelic injection of freshly grown live bacteria into L5 larvae of S. exigua (Table 1). All bacterial treatments exhibited insecticidal activities. However, their insecticidal activities were different, with LD50 ranging from 168.8 (P. temperata temperata: ‘Ptt’) to 641.3 (X. ehlersii: ‘Xe’) CFU/larva.

To analyze variations in bacterial insecticidal activities, their secondary metabolites were extracted from bacterial culture broth with four different organic solvents: hexane (‘HEX’), ethyl acetate (‘EAX’), chloroform (‘CX’), and butanol (‘BX’) extracts. These organic extracts showed variations in insecticidal activities, with LD50 values ranging from 92.4 (BX from X. hominickii: ‘Xh’) to 787.6 (HEX from ‘Xe’) μg/larva (Table S1). Insecticidal activities of bacteria and their extracts exhibited high positive correlations (Fig. 1): bacterial toxicity with HEX ($r = 0.954; P < 0.0001$), EAX ($r = 0.938; P < 0.0001$), CX ($r = 0.967; P < 0.0001$), and BX ($r = 0.967; P < 0.0001$).

Comparative analysis of bacterial extracts for insect immunosuppression

Variations in insecticidal activities among entomopathogenic bacteria might be caused by their differential immunosuppressive capabilities against target insects. To test this hypothesis, effects of bacterial extracts on cellular immune responses were assessed using hemocyte-
spreading behaviour and nodulation assays after bacterial infection. All extracts significantly ($p < 0.05$) inhibited the hemocyte-spreading behavior (Fig. 2a) and nodulation (Fig. 2b). Immunosuppression was significantly ($p < 0.05$) rescued by the addition of arachidonic acid (a catalytic product of PLA$_2$), suggesting that the immunosuppressive activities of these bacterial extracts were different among bacterial species. When bacterial insecticidal activities were compared with immunosuppressive activities, these two parameters showed high positive correlations for all extracts (Fig. 2c).

To determine whether immunosuppressive activities of these bacterial extracts were caused by inhibition of insect PLA$_2$, these extracts were incubated with PLA$_2$ extract obtained from _S. exigua_ hemocytes. All bacterial extracts significantly inhibited sPLA$_2$ activity, although they showed variations in inhibiting sPLA$_2$ (Fig. 3a). For cPLA$_2$, some extracts failed to inhibit its enzyme activity (Fig. 3b). Compared to HEX and CX, EAX and BX appeared to show higher inhibitory activities. Ptt and Xh extracts showed significantly higher inhibitory activities than the other bacterial extracts. There were positive correlations between bacterial insecticidal activities and PLA$_2$ inhibition except HEX against cPLA$_2$ (Fig. 3c).

**Prediction of virulent secondary metabolites produced by *Xenorhabdus* and *Photorhabdus***

Immunosuppressive activities of bacterial extracts suggested that their secondary metabolites contained inhibitory compounds. To predict functional secondary metabolites, all extracts were assessed by TLC to confirm the presence of compounds. These compounds were then analyzed by GC-MS (Figure S1). Predicted compounds of four extracts for each bacterial species were combined and resulting 182 compounds were compared between bacterial isolates (Table S2). In addition to different compositions among bacterial isolates, the total number of secondary metabolites varied from 32 compounds of _X. nematophila_ SK1 to 63 compounds of Ptt. Interestingly, there was a significant ($p < 0.05$) negative correlation between the total number of secondary metabolites and target insect immune responses measured by nodulation, sPLA$_2$ activity, or cPLA$_2$ (Fig. 4a). In addition, if bacteria had more secondary metabolites, they exhibited higher insecticidal activities.

To select virulent bacterial compounds, two most potent bacteria, Ptt and Xh, were compared with the least potent Xe for their secondary metabolites (Fig. 4b). Ptt and Xh shared over 44% metabolites. Almost 82% metabolites of Xe were detected in the culture broth of Ptt or Xh. Thus, Ptt- (29 compounds) or Xh- (23 compounds) specific compounds and overlapping (17 compounds) compounds were chosen as possible virulent compounds (Table 2).

**Validation of virulent secondary metabolites for their immunosuppression and insecticidal activities**

Twelve compounds were selected from 70 virulent candidates produced by Ptt or Xh (Table S3). As a reference, one compound (2-mercaptobenzothiazole: ‘MT’) was randomly selected from metabolites produced by Xe as well as Ptt and Xh. All 12 virulent compounds highly suppressed hemocytic nodulation in response to bacterial infection (Fig. 5a). MT also suppressed immune responses, although it exhibited much lower inhibitory activity than those 12 virulent compounds. Compounds that showed the highest inhibitory activities were indole (‘IND’), ethoxymethoxyphenol (‘EMP’), and dimethylhydroxynor- withancy (‘DHF’). These 12 virulent compounds significantly ($p < 0.05$) inhibited sPLA$_2$ activity (Fig. 5b) and cPLA$_2$ activity (Fig. 5c) whereas MT did not. IND, EMP, and DHF also showed high inhibitory activities against PLA$_2$. These 12 virulent compounds also showed high insecticidal activities (Table 3). In contrast, MT had relatively low toxicities to the test insect species.

### Table 1 Insecticidal activities of 14 entomopathogenic bacteria (EPB) of *Xenorhabdus* and *Photorhabdus* against L5 larvae of _S. exigua_. Larvae were hemocoelically injected with different doses of freshly cultured bacteria. Before injection, larvae were surface sterilized with 70% ethanol. For each test dose, 10 larvae were used with three replications.

| EPB* | LD$_{50}$ (cfu/larva) (95% CI) | Slope ± SE | df | $\chi^2$ |
|------|------------------------|-----------|----|--------|
| Ptt  | 168.8 (110.4–318.3)     | 0.87 ± 0.27 | 1  | 0.817  |
| Xh   | 179.2 (156.9–345.6)     | 0.77 ± 0.29 | 2  | 0.953  |
| Xn F | 216.9 (114.6–406.8)     | 0.82 ± 0.27 | 2  | 0.906  |
| Xb   | 222.4 (120.3–432.7)     | 0.77 ± 0.28 | 2  | 0.835  |
| Xn M | 244.3 (147.5–480.4)     | 0.87 ± 0.26 | 2  | 0.959  |
| Pl 193| 244.3 (128.6–487.3)     | 0.87 ± 0.26 | 2  | 0.959  |
| Pl laum| 274.8 (150.3–512.6)   | 0.74 ± 0.29 | 2  | 0.985  |
| Xn SK2| 274.9 (151.4–523.2)   | 0.74 ± 0.29 | 2  | 0.985  |
| Pl thra| 282.87 (148.8–551.4)  | 0.97 ± 0.24 | 2  | 0.911  |
| Xn 12,145| 360.7 (201.7–680.4) | 0.85 ± 0.26 | 2  | 0.849  |
| Xn K1 | 550.9 (296.8–1079.8)   | 0.64 ± 0.32 | 1  | 0.879  |
| Xn SK1| 552.3 (294.2–1089.5)   | 0.64 ± 0.32 | 2  | 0.987  |
| Xp   | 556.9 (289.5–1069.5)   | 0.64 ± 0.32 | 1  | 0.784  |
| Xe   | 641.3 (346.4–1192.7)   | 0.66 ± 0.31 | 1  | 0.964  |

*EPBs include *Photorhabdus temperata* Ssp. *temperata* ANU101 (Ptt), *Xenorhabdus hominickii* ANU101 (Xh), *X. nematophila* France (XnF), *X. bovenii* (Xb), *X. nematophila* Mexico (XnM), *Photorhabdus* luminescens KACC12123 (Pl 193), *P. luminescens* subsp. laumondii KACC12283 (Pl laum), *X. nematophila* SK2 (XnSK2), *P. luminescens* subsp. *thracensis* KACC12284 (Pl thra), *X. nematophila* KACC12145 (Xn12145), *Xenorhabdus nematophila* K1 (XnK1), *X. poinarii* (Xp), and *X. ehlersii* KSY (Xe)
However, IND, EMP, and DHF exhibited high insecticidal activities, with LD$_{50}$ ranging from 4.29 to 5.12 μg/larva.

**Discussion**

Bacterial species in genera *Xenorhabdus* and *Photorhabdus* can produce various secondary metabolites to maintain their mutualistic symbiosis with host entomopathogenic nematodes [24]. Some of metabolites are required for their colonization to specific nematode hosts while others are crucial for pathogenesis specifically targeting insect’s immune system [3]. This study assessed all metabolites extracted with four organic solvents from bacterial culture broth of *Xenorhabdus* and *Photorhabdus* to identify their virulent secondary metabolites against insect immune responses.

Organic extracts of bacterial culture broth of seven different species classified in *Xenorhabdus* and *Photorhabdus* showed immunosuppressive and insecticidal activities. However, pathogenic activities of these extracts were different among 14 bacterial isolates and highly correlated with their bacterial insecticidal activities. These results suggest that these bacteria can induce immunosuppression of target insects via their secondary metabolites, supporting the hypothesis that immunosuppression is required for bacterial pathogenicity. Protein toxins that also induce the immunosuppression and finally kill target insects are known in *Xenorhabdus* and *Photorhabdus* [25]. However, acute attack by a target insect’s immune system should be avoided and actively suppressed by the secondary metabolites [26].

Immunosuppression of target insects by *Xenorhabdus* and *Photorhabdus* infection is caused by inhibition of PLA$_2$ by bacterial secondary metabolites [10]. The first identified PLA$_2$ inhibitor produced by bacteria was benzylideneacetone (BZA) produced by *X. nematophila* [27]. Subsequently, eight different PLA$_2$ inhibitors were identified from bacterial culture broths of *X. nematophila* and *P. temperata* [13]. On the other hand, these PLA$_2$ inhibitors induced apoptosis-associated cytotoxicity and insecticidal activities [28]. This explains why bacterial metabolites containing PLA$_2$ inhibitors in our current study showed insecticidal activities. Indeed, the degree of PLA$_2$ inhibition by bacteria has been found to be highly correlated with bacterial pathogenicity [15]. These findings suggested that bacterial metabolites that inhibited PLA$_2$ activities in this current study contained virulent secondary metabolites.
Virulent secondary metabolites produced by *Xenorhabdus* and *Photorhabdus* were chosen based on comparative analysis of secondary metabolites among bacterial isolates. GC-MS analyses for bacterial culture broths of 14 isolates predicted 182 compounds. Most of them were phenol derivatives, tryptophan derivatives, peptides, and fatty acid derivatives including secondary metabolites synthesized by NRPS-PKS [4]. In general, predicted and identified compounds from NRPS-PKS of *Xenorhabdus* and *Photorhabdus* genomes are associated with bacterial pathogenicity [19]. This is supported by the high correlation between the number of predicted compounds in each bacterial isolate and bacterial pathogenicity found in the current study. This also suggests that these compounds predicted by GC-MS might contain virulent secondary metabolites. To select virulent compounds, predicted metabolites of highly pathogenic bacterial isolates (Ptt and Xh) were compared with those

**Fig. 2** Suppression of cellular immune responses by four organic extracts of 14 bacterial isolates and its correlation with bacterial pathogenicity. Bacteria used in this assay were: *Photorhabdus temperata* Subsp. *temperata* ANU101 (Ptt), *Xenorhabdus hominickii* ANU101 (Xh), *X. nematophila* K1 (XnK1), *X. ehlersii* KSY (Xe), *X. nematophila* SK1 (XnSK1), *X. nematophila* SK2 (XnSK2), *Photorhabdus luminescens* KACC12123 (Pl 193), *P. luminescens* subsp. *laumondii* KACC12283 (Pl laum), *P. luminescens* subsp. *thraccensis* KACC12284 (Pl thra), *X. nematophila* KACC12145 (Xn12145), *X. nematophila* Mexico (XnM), *X. nematophila* France (XnF), *X. bovienii* (Xb), and *X. poinarii* (Xp). Their cultured broths were extracted with four different organic solvents: hexane (HEX), ethyl acetate (EAX), chloroform (CX), and butanol (BX). Effects of organic extracts on hemocyte-spreading behavior. For each treatment, three independently prepared hemocyte mixtures were used. To determine the spreading behavior, 100 hemocytes were randomly chosen. Effects of organic extracts on hemocyte nodulation in response to bacterial challenge. Each LS larva of *S. exigua* was injected with bacterial extract (10 μg/larva) along with heat-killed *E. coli* (4 × 10⁴ cells). For each treatment, three replications were used with five larvae per replication. Arachidonic acid (AA, a catalytic product of PLA₂) was used to rescue the inhibition. Different letters above standard deviation bars indicate significant differences among means at Type I error = 0.05 (LSD test). Correlations (r) between insecticidal activities of bacterial extracts (Fig. 1) and their immunosuppressive activities. Lines represent the best-fit regression.

*Xenorhabdus* and *Photorhabdus* genomes are associated with bacterial pathogenicity [19]. This is supported by the high correlation between the number of predicted compounds in each bacterial isolate and bacterial pathogenicity found in the current study. This also suggests that these compounds predicted by GC-MS might contain virulent secondary metabolites. To select virulent compounds, predicted metabolites of highly pathogenic bacterial isolates (Ptt and Xh) were compared with those...
of a low pathogenic isolate (Xe). The comparison for 103 total compounds resulted in the selection of 70 compounds, including Ptt-specific, Xh-specific, and Ptt + Xh common metabolites. Among 70 virulent candidates, 12 randomly chosen compounds showed high immuno-suppressive and insecticidal activities, validating the prediction of these 70 virulent secondary metabolites of Xenorhabdus and Photorhabdus. This analysis did not include 80 compounds (182 compounds from 14 total isolates – 103 compounds of 3 isolates used for the comparison) because other bacterial isolates were less virulent than Ptt and Xh. Among 12 virulent compounds, three (IND, EMP, and DHF) were highly potent in inhibiting insect immune responses, exhibiting high insecticidal activities. These compounds are not likely to be the secondary metabolites produced from NRPS-PKS. Although secondary metabolites derived from NRPS-PKS contribute to induce the bacterial pathogenicity as mentioned above, non-NRPS-PKS metabolites may also play crucial role in expressing insecticidal activity of the bacteria. This is supported by the fact that a mutant X. szen-tirmaili in a specific phosphopantethienyl transferase, which activates the carrier protein domain of PKS, NRPS, and fatty acid synthase [29], loses the production.
of NRPS-PKS metabolites but exhibits insecticidal virulence [30]. IND has been found to be a PLA$_2$ inhibitor from entomopathogenic bacteria [13]. Unlike tryptophan derivatives including indole, EMP is a phenol derivative that shares structural similarities with other sPLA$_2$ inhibitors such as 4-bromophenacyl bromide and BZA. DHF is a furanone and a relatively new chemical compound that can inhibit PLA$_2$ among bacterial metabolites. Derivatives containing these three different skeletons need to be compared for their activities to inhibit insect PLA$_2$ to develop potent immunosuppressants. In summary, this study newly proposes other PLA$_2$ inhibitors including EMP and DHF.

**Conclusions**

Bacterial isolates of *Xenorhabdus* and *Photobacterium* were pathogenic by suppressing insect immune responses via PLA$_2$ inhibition using their secondary metabolites. Such immunosuppression was highly correlated with insecticidal activity of bacteria. Based on this correlation, 70 virulent compounds were predicted
### Table 2
Prediction of 70 virulent secondary metabolites derived from *X. hominickii* (*Xh*) and *P. temperata* (*Ptt*). These metabolites were predicted from GC-MS analysis of extracts of bacterial culture broths.

| Group* | No | Compounds                                      |
|--------|----|------------------------------------------------|
| *Ptt + Xh* | 1  | 1-Butanamine, N-butyl-                         |
|         | 2  | Benzyl alcohol                                 |
|         | 3  | 5-Thiazoleethanol, 4-methyl-                   |
|         | 4  | Indole                                         |
|         | 5  | Benzeneethanol, 4-hydroxy-                     |
|         | 6  | Phthalimide                                    |
|         | 7  | o-Cyanobenzoic acid                            |
|         | 8  | Acetamide, N-(2-phenylethyl)-                  |
|         | 9  | Indole-3-pyruvic acid                          |
|         | 10 | Tryptophol                                     |
|         | 11 | 1H-Indole-3-acetic acid, hydrazide             |
|         | 12 | 2-Mercaptophenol                               |
| *Ptt*   | 1  | Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-  |
|         | 2  | 1H-Indole-3-ethanol, acetate (ester)           |
|         | 3  | 2-Dodecen-1-yl (−) succinic anhydride          |
|         | 4  | Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- |
|         | 5  | 2,5-Piperazinedione, 3-(phenylmethyl)-         |
| *Xh*    | 1  | 1-Hexanol, 2-ethyl-                            |
|         | 2  | Pyrazine, 3-ethyl-2,5-dimethyl-                |
|         | 3  | Benzeneethanamine                              |
|         | 4  | Hexanoic acid, 5-oxo-, ethyl ester            |
|         | 5  | 1,1-Disobutoxy-isobutane                      |
|         | 6  | Formamide, N, N-dibutyl-                      |
|         | 7  | Cyclohexasiloxane, dodecamethyl-              |
|         | 8  | Butanoic acid, butyl ester                    |
|         | 9  | Propanoic acid, 2-methyl-, butyl ester        |
|         | 10 | 2-Tetradecene, (E)-                           |
|         | 11 | 3-Ethoxy-4-Methoxyphenol                      |
|         | 12 | 7,9-Dimethyl-1,4-dioxo-7,9-diazacycloundecane-3-thione |
|         | 13 | Heptadecane, 2,6-dimethyl-                    |
|         | 14 | 1H-Indole-3-acetic acid, methyl ester         |
|         | 15 | 1H-Indene, 2-butyl-5-hexyloctahydro-          |
|         | 16 | L-Proline, N-valeryl-, decyl ester            |
|         | 17 | Stannane, tetraethyl-                         |
|         | 18 | Fluorene, 4-(1,2-dihydroxyethyl)-             |
|         | 19 | 1-Eicosene                                    |
|         | 20 | Nonadecanenitrile                             |
|         | 21 | Heptadecanoic acid, 14-methyl-, methyl ester  |
|         | 22 | E-8-Methyl-9-tetradecen-1-ol acetate          |
|         | 23 | Pentanamide, N-[2-(indol-3-yl)] ethyl-        |
| *Ptt*   | 1  | 4-Ethylamino-n-butylamine                     |
|         | 2  | 2,5-Dimethyl-4-hydroxy-3(2H)-furanone         |
|         | 3  | Octanoic acid                                 |
by comparative analysis of secondary metabolites between high and low pathogenic bacteria.

**Methods**

**Insect and bacteria**

Larvae of *Spodoptera exigua* were reared at temperature of 25 ± 2 °C and relative humidity of 65 ± 5% under 16 h light and 8 h dark condition with an artificial diet [31]. Under these conditions, larvae underwent five instars (L1-L5). Adults were provided with 10% sucrose solution. For bioassays to determine bacterial pathogenicity, larvae were taken from a cohort.

Ten strains of *Xenorhabdus* and four strains of *Photorhabdus* were collected from previous stocks: \[X. nematophila \text{K1 (‘XnK1’)}\] [32], \[X. hominickii ANU101 (‘Xh’)] [16], \[X. ehlersii KSY (‘Xe’)\] [33], \[*Photorhabdus temperata* Ssp. *temperata* ANU101 (‘Ptt’)] [34], \[*X. nematophila* SK1 (‘XnSK1’) and *X. nematophila* SK2 (‘XnSK2’)\] [15], Korean Agricultural Culture Collection (KACC, RDA, Jeonju, Korea) \[*Photorhabdus luminescens* KACC12123 (‘Pl 193’), \[*P. luminescens* subsp. *laumondii* KACC12283 (‘Pl laum’), \[*P. luminescens* subsp. *thracensis* KACC12284 (‘Pl thra’), \[*X. nematophila* KACC12145 (‘Xn12145’), \[*X. nematophila* Mexico (‘XnM’), \[*X. nematophila* France (‘XnF’), \[*X. bovienii* (‘Xb’), and \[*X. poinarii* (‘Xp’)\]. These bacteria were grown in tryptic soy broth (TSB: Difco, Sparks, MD, USA) for 48 h at 28 °C with shaking at 180 rpm [16, 32]. *Escherichia coli* Top 10 was purchased from Invitrogen (Carlsbad, CA, USA) and cultured overnight in Luria-Bertani (LB) medium at 37 °C with shaking at 180 rpm. For immune challenge experiment, heat killed (80 °C for 10 min) *E. coli* were used. Their cell number was counted using a hemocytometer (Neubauer improved bright line, Cat. No. 0640010, Superior Marienfeld, Lauda-Konigshofen, Germany) under a phase contrast microscope (BX41, Olympus, Tokyo, Japan). Different bacterial concentrations were prepared through serial dilution in sterilized deionized distilled water.

### Table 2 Prediction of 70 virulent secondary metabolites derived from *X. hominickii* (‘Xh’) and *P. temperata temperata* (‘Ptt’). These metabolites were predicted from GC-MS analysis of extracts of bacterial culture broths (Continued)

| Group\(^a\) | No | Compounds |
|---|---|---|
| 4 |  | Benzothiazole |
| 5 |  | 1,2-Ethanediol, 1-phenyl- |
| 6 |  | n-Decanoic acid |
| 7 |  | Phenol, 2,6-bis(1,1-dimethylethyl)- |
| 8 |  | Dodecanoic acid |
| 9 |  | 1-Pentadecene |
| 10 |  | 1H-Benzimidazole, 2-(methylthio)- |
| 11 |  | Propanamide, 2-amino-3-(3-indolyl)- |
| 12 |  | Hexadecane, 7,9-dimethyl- |
| 13 |  | 1-Hexadecan-2-yl- |
| 14 |  | Dicyclohexylisulphide |
| 15 |  | 1-Nonadecene |
| 16 |  | Propanamide, 2,2,3,3,3-pentafluoro-N-(2-phenylethyl)- |
| 17 |  | 1,13-Tetradecadien-3-one |
| 18 |  | Hexadecanoic acid, methyl ester |
| 19 |  | Diethyl Phthalate |
| 20 |  | E-15-Heptadecenal |
| 21 |  | 3-Phenylbicyclo (3.2.2) nona-3,6-dien-2-one |
| 22 |  | E-11-Methyl-12-tetradecen-1-ol acetate |
| 23 |  | 2-Methyl-E-7-octadecene |
| 24 |  | Octadecanenitrile |
| 25 |  | Pyrene, 4-methyl- |
| 26 |  | 9-Octadecenamide, (Z)- |
| 27 |  | Di-n-octyl phthalate |
| 28 |  | Zinc, bis (dimethylethamidioato-S, S’)-, (T-4)- |
| 29 |  | Zinc dibutylthiociarbamate |

\(^a\)Groups are classified by bacterial metabolites from both Ptt and Xh (‘Ptt + Xh’), only Xh (‘Xh’), and only Ptt (‘Ptt’).
Chemicals

Arachidonic acid (5,8,11,14-eicosatetraenoic acid; AA), tryptophol (TPL), indole (IND), indole-3-acetic acid hydrazide (IAAH), o-cyanobenzoic acid (CBA), 2-ethyl-1-hexanol (EH), 2-mercaptophenol (MP), and 2-mercaptobenzothiazole (MT) were purchased from Sigma-Aldrich Korea (Seoul, Korea) and dissolved in dimethyl sulfoxide (DMSO). 1,2-bis (heptanoylthio) phosphatidylcholine (PC) was used as secretory PLA$_2$ (sPLA$_2$) substrate while arachidonoyl thio-PC was used as cellular PLA$_2$ (cPLA$_2$) substrate. These substrates were purchased from Cayman Chemical (Ann Arbor, MI, USA). Benzyl alcohol (BA) and benzeneethanol-4-hydroxy (BH) were purchased from Alfa Aesar China (Shanghai, China). 3-ethoxy-4-methoxyphenol (EMP), indole-3-acetic acid hydrazide (IAAH), indole (IND), 2-mercaptophenol (MP), 2-mercaptobenzothiazole (MT), N-(2-phenylethyl) acetamide (NPA), 1-phenyl-1,2-ethanediol (PE), and tryptophol (TPL). These compounds are classified as bacterial metabolites synthesized by both Ptt and Xh (Ptt + Xh), only Xh (Xh), only Ptt (Ptt), and common to Ptt, Xh, and Xe (Xe).

Fractionation of bacterial culture broth and TLC analysis

All bacterial isolates were cultured individually in TSB (1 L) at 28 °C with shaking at 180 rpm. After 48 h,
Table 3 Insecticidal activities of virulent secondary metabolites of *P. temperata* temperata (Ptt) and *X. hominickii* (Xh) against *S. exigua*. Virulent metabolites were compared with the reference compound produced by a less virulent *X. ehlersii* (Xe). Bioassays were conducted by hemocoelic injection to L5 larvae. For each treatment dose, 10 larvae were used.

| Groupa | Compoundb | LD50 (μg/larva) (95% CI) | Slope ± SE | df | χ² |
|--------|-----------|--------------------------|------------|----|----|
| Ptt + Xh | IND | 5.12 (2.51–10.2) | 0.50 ± 0.41 | 3 | 0.813 |
| CBA | 8.52 (2.16–16.4) | 0.47 ± 0.44 | 3 | 0.774 |
| NPA | 12.79 (6.1–24.5) | 0.32 ± 0.59 | 3 | 0.953 |
| TPL | 7.44 (3.8–34.9) | 0.44 ± 0.47 | 3 | 0.905 |
| IAAH | 14.14 (7.6–27.6) | 0.31 ± 0.61 | 3 | 0.944 |
| MP | 15.15 (7.9–30.3) | 0.29 ± 0.66 | 3 | 0.993 |
| BA | 17.93 (3.9–34.7) | 0.31 ± 0.63 | 3 | 0.940 |
| BH | 22.47 (17.4–62.9) | 0.35 ± 0.58 | 3 | 0.701 |
| Ptt | DHF | 4.94 (3.2–8.7) | 0.49 ± 0.42 | 3 | 0.718 |
| PE | 24.28 (12.6–47.5) | 0.42 ± 0.49 | 3 | 0.819 |
| Xh | EH | 8.93 (4.8–17.5) | 0.43 ± 0.47 | 3 | 0.492 |
| EMP | 4.29 (2.1–8.4) | 0.36 ± 0.53 | 3 | 0.926 |
| Xe | MT | 38.75 (20.0–75.6) | 0.38 ± 0.54 | 3 | 0.691 |

aGroup is classified by bacterial compounds specific to (1) both Ptt and Xh (Ptt + Xh), (2) only Ptt (Ptt), (3) only Xh (Xh), and (4) common to Ptt, Xh, and Xe (Xe).
bCompound acronyms are: benzyl alcohol (BA), benzeneethanol-4-hydroxy (BHE), o-cyanobenzoic acid (CBA), 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DHF), 2-ethyl-1-hexanol (EH), 3-ethoxy-4-methoxyphenol (EMP), indole-3-aceticacid hydrazide (IAAH), indole (IND), 2-mercaptobenzothiazole (MT), N-2-phenethylethyl acetamide (NPA), 1-phenyl-1,2-ethanediol (PE), and tryptophol (TPL).

bacterial pellets were separated from supernatant after centrifuging cultured broth at 10,000 × g for 20 min at 4 °C. The resulting supernatant was subjected to fractionation of secondary metabolites by successively adding four different organic solvents (hexane, ethyl acetate, chloroform, and butanol) as described previously by Mollah et al. [35]. Resulting extracts were resuspended in methanol (0.4 mg/mL) and further diluted with DMSO or methanol to desired concentrations based on experimental purposes. During each fractionation step, the metabolite extraction was subjected to thin layer chromatography (TLC) by spotting it on a silica gel plate (20 × 20 cm: Merck, Darmstadt, Germany). A mixture of chloroform, methanol, and acetic acid (7:2.5:0.5, v/v) was used as an eluent. Spots in the silica gel plate were visualized and marked under a fluorescence analysis cabinet (Spectroline, CM-10, Westbury, NY, USA).

Gas chromatography coupled with mass spectrophotometer (GC-MS) analysis

GC (7890B, Agilent Technologies, Santa Clara, CA, USA) equipped with MS (5977A Network, Agilent Technologies) was used for the prediction of the bacterial extracts in the methanol resuspension. It was performed as described by Mollah et al. [35]. An HP 5 MS column (non-polar column, Agilent Technologies) was used for GC. Helium was used as carrier gas at a flow rate of 1 mL/min. Split mode of injection with split ratio 10:1 was followed at 200 °C. The initial temperature was 100 °C for 3 min. It was raised to 300 °C at 5 °C/min. For recording mass spectra, EI mode at 70 eV with a scanning range of 33–550 m/z was used. Mass spectra were used for identification of purified samples using NIST11 database (U.S. Department of Commerce, Gaithersburg, MD, USA) and literature data (http://nistmassspeclibrary.com).

Hemocyte-spreading behavior assay

Hemolymph (~ 250 μL) was collected from L5 larvae of *S. exigua* by cutting prolegs. It was mixed with 750 μL ACB. The hemolymph suspension was centrifuged at 800 × g for 5 min and 800 μL supernatant was mixed with filter-sterilized 200 μL TC100 insect culture medium (Hyclone, Daegu, Korea). This hemocyte suspension (9 μL) and each bacterial metabolite (1 μL) were mounted on a glass slide to assess hemocyte-spreading behavior. For rescue experiment with AA (10 μg/μL in DMSO), 8 μL of cell suspension, 1 μL of bacterial metabolite, and 1 μL of AA were placed on a glass slide. After incubating the mixture at room temperature under darkness for 40 min, hemocytes were observed under a phase contrast microscope (Olympus) at 800 × magnification. As control, the dilution solvent (DMSO) of metabolites was used. Spread hemocytes were recognized based on cytoplasmic extension out of cell boundary. Each treatment was replicated three times with separately prepared suspension mixture. In each replication, 100 hemocytes were randomly chosen to assess hemocyte-spreading behavior.

Nodulation assay

Hemocyte nodule formation was observed using 3 days old L5 larvae of *S. exigua* after injecting 3 μL of overnight grown *E. coli* Top 10 (10⁴ cells/larva) and 2 μL of DMSO to the hemocoeel through prolegs using a micro-syringe (Hamilton, Reno, NV, USA). To assess any inhibitory effect of bacterial extracts or secondary metabolites, 1 μL (1 μg/μL in DMSO) of test sample was co-injected with 3 μL of *E. coli* and 1 μL of DMSO. For AA rescue experiment, 1 μL of AA (10 μg/μL in DMSO) was co-injected with 1 μL of bacterial metabolite and 3 μL of *E. coli*. After incubation at 25 °C for 8 h, injected larvae were dissected to count melanized nodules under a stereomicroscope (Stemi SV11, Zeiss, Jena, Germany) at 50 × magnification. Each treatment was replicated three times using five larvae per replication.
PLA\(_2\) enzyme assay
PLA\(_2\) activity was assessed using processes previously described by Vatanparast et al. [36]. Briefly, enzymes were extracted after grinding hemocytes of L5 larvae of \(S.\) exigua with PBS followed by centrifugation at 12, 500xg for 5 min. The resulting supernatant containing the cytosol and membrane mixture was used as enzyme source. The reaction mixture (225 \(\mu\)L) contained 10 \(\mu\)L of PLA\(_2\) enzyme, 10 \(\mu\)L of Ellman's reagent (5,5-di-thiobis-(2-nitrobenzoicacid)), 5 \(\mu\)L of test inhibitor, and 200 \(\mu\)L of sPLA\(_2\) or cPLA\(_2\) substrate. DMSO was used instead of test inhibitor as control. Change in absorbance of reaction product was measured at wavelength of 405 nm using a microplate reader (Victor, PerkinElmer, Waltham, MA, USA). Each treatment was replicated three times with biologically independent samples.

Bacterial pathogenicity test
To determine insecticidal activities of 14 bacterial isolates, bacterial cells after 24 h of culture were centrifuged at 10,000xg for 2 min at 4 °C. Cell pellet was resuspended in PBS for injection. Bacterial suspension was diluted with PBS to obtain different concentrations (0, \(10^1\), \(10^2\), \(10^3\), \(10^4\) and \(10^5\) colony-forming unit (cfu)/ larva) and injected to hemocoel of L5 \(S.\) exigua larvae using a microsyringe. Before injection, larvae were surface sterilized with 70% ethanol. Bacterial dose was measured after plating the suspension onto LB agar medium face sterilized with 70% ethanol. Bacterial dose was measured after plating the suspension onto LB agar medium. As control, \(E.\) coli was used. Injected larvae were incubated at room temperature with sufficient diet. Larvae were considered to be dead if there was no movement upon touching. Each treatment consisted of three replications using 10 larvae for each replication.

Toxicity tests of bacterial metabolites and synthetic compounds
To determine toxicities of bacterial extracts and predicted metabolites, hemocoelic injection was performed. Dried bacterial extracts and metabolites were weighed and dissolved in DMSO to prepare different concentrations (0.001, 0.01, 0.1, 1 and 10 \(\mu\)g/\(\mu\)L) for toxicity assays. A 10 \(\mu\)L microsyringe was used for hemocoelic injection. Each test sample (3 \(\mu\)L) was injected to L5 larvae of \(S.\) exigua. DMSO was injected as control. Treated larvae were placed in 9 cm diameter dishes and incubated at room temperature. Mortality was determined every 24 h for 96 h after treatment. Larvae were considered as dead if there was no movement upon touching. Each test concentration was replicated three times using 10 larvae per replication.

Statistical analysis
All assay data for continuous variables were subjected to one-way analysis of variance (ANOVA) using PROG GLM in SAS program [37]. For ANOVA, mortality data were subjected to arcsine transformation. Means were compared with the least significant difference (LSD) test at 0.05 level of Type I error. Median lethal dose (LD\(_{50}\)) was calculated using EPA Probit Analysis Program, ver. 1.5 (U.S. Environmental Protection Agency, Washington, D.C., USA).

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12866-020-02042-9.

Additional file 1: Table S1. Insecticidal activities of four organic extracts of culture broth of 14 entomopathogenic bacteria (EPB) of \(Xenorhabdus\) and \(Photorhabdus\) against L5 larvae of \(S.\) exigua. The bioassay was carried out by injecting 3 \(\mu\)L of bacterial culture broth extracts from different concentrations into the hemocoel of L5 larva. Each treatment dose used 10 larvae and each treatment replicated three times.

Table S2. GC-MS prediction of secondary metabolites in organic extracts of bacterial culture broth from 14 species of \(Xenorhabdus\) and \(Photorhabdus\).

Table S3. Secondary metabolites predicted from \(Photorhabdus\) temperata temperata (Ptt), \(Xenorhabdus\) hominickii (Xh) and \(Xenorhabdus\) ehlersii (Xe) which were used for biological activity analysis.

Figure S1. Chromatograms from the GC-MS analysis of the organic extracts from different concentrations into the hemocoel of L5 \(S.\) exigua larvae using a microsyringe. Before injection, larvae were surface sterilized with 70% ethanol. Bacterial dose was measured after plating the suspension onto LB agar medium followed by culturing at 28°C for 48 h. Mortality was counted at 24 h after bacterial injection. As control bacterial treatment, \(E.\) coli was used. Injected larvae were incubated at room temperature with sufficient diet. Larvae were considered to be dead if there was no movement upon touching. Each treatment consisted of three replications using 10 larvae for each replication.

Abbreviations
AA: Arachidonic acid; ACB: Anticoagulant buffer; ANOVA: Analysis of variance; BA: Benzyl alcohol; BZA: Benzylideneacetone; BH: Benzeneethanol-4-hydroxy; CBA: Cyanobenzoic acid; CFU: Colony-forming unit; DHF: Dimethylhydroxyfurane; DMSO: Dimethyl sulfoxide; EH: Ethyl-1-hexanol; EMP: Ethoxymethylphenoxphenol; IAAH: Indole-3-acetic acid hydrazide; IND: Indole; LD\(_{50}\): Median lethal dose; LSD: Least significant difference; MP: Mecaptophenol; MT: Mercaptophenothiazole; NRPS: Non-ribosomal peptide synthetase; PC: Phosphatidylcholine; PKS: Polyketide synthase; PLA\(_2\): Phospholipase A\(_2\); Lrp: Leucine-responsive protein; NPA: N-(2-phenylethyl) acetamide; PE: Phenyl-1,2-ethanediol; PO: Phenoxyacetate; Ptt: \(Photorhabdus\) temperata temperata; TPL: Tryptophol; TSB: Tryptic soy broth; Xh: Xenorhabdus hominickii; Xe: Xenorhabdus ehlersii; Xb: \(Xenorhabdus\) bovienii; Xp: \(Xenorhabdus\) poinarii

Acknowledgements
The authors thank Youngim Song for supplying all materials and Miltan Chandra Roy for rearing test insects.
Authors’ contributions
YK designed experiments and revised the manuscript draft. MIM performed all experiments and data analyses. MIM prepared the manuscript. All authors have read and approved the manuscript.

Funding
This work was supported by a grant (No. 2017R1A1B1002191) of the National Research Foundation (NRF) funded by the Ministry of Science, ICT and Future Planning, Republic of Korea.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information.

Ethics approval and consent to participate
Not Applicable.

Consent for publication
Not Applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 21 August 2020 Accepted: 10 November 2020
Published online: 23 November 2020

References
1. Akhurst RJ. Neoseptica species: specificity of association with bacteria of the genus Xenorhabdus. Exp Parasitol. 1983;55:258–63.
2. Shapiro-Ilan DI, Han R, Dolinksi C. Entomopathogenic nematode production and application technology. J Nematol. 2012;44:206–17.
3. Goodrich-Blair H, Clarke DJ. Mutualism and pathogenesis in Xenorhabdus and Photorhabdus: two roads to the same destination. Mol Microbiol. 2007;64:260–8.
4. Tobias NJ, Wolff H, Djahanchip B, Grundmann F, Kronenweth M, Shi Y, et al. Natural product diversity associated with the nematode symbionts Photorhabdus and Xenorhabdus. Nat Microbiol. 2017;2:1676–85.
5. Mollah MI, Roy MC, Choi D, Hasan MA, Al Baki MA, Yeom HS, et al. Variations of indole metabolites and NRPS-PKS loci in two different virulent strains of Xenorhabdus hominickii. Front Microbiol. 2020;11:5000.
6. Sajjadian SM, Kim Y. Dual oxidase-derived reactive oxygen species against Bacillus thuringiensis and its suppression by eicosanoid biosynthesis inhibitors. Front Microbiol. 2020;11:528.
7. Park Y, Herbert EE, Cowles CE, Cowles KN, Menard ML, Orchard SS, et al. Clonal variation in Xenorhabdus nematophila virulence and suppression of Manduca sexta immunity. Cell Microbiol. 2007;9:465–56.
8. Sergeant M, Baxter L, Jarrett P, Shaw E, Ousley M, Winstanley C, et al. Identification, typing, and insecticidal activity of Xenorhabdus isolates from entomopathogenic nematodes in United Kingdom soil and characterization of the xpt toxin loci. Appl Environ Microbiol. 2006;72:3895–907.
9. Shrestha S, Kim Y. An entomopathogenic bacterium, Xenorhabdus nematophila, inhibits hemocyte phagocytosis of Spodoptera exigua by inhibiting phospholipase A2. J Invertebr Pathol. 2007;96:64–70.
10. Kim Y, Cho JD, Park Y. Two groups of entomopathogenic bacteria, Photorhabdus and Xenorhabdus, share an inhibitory action against phospholipase A2 to induce host immunodepression. J Invertebr Pathol. 2005;89:258–64.
11. Mouchlis VD, Dennis EA. Phospholipase A2 catalysis and lipid mediator lipidomics. Biochim Biophys Acta. 2019;1864:766–71.
12. Kim Y, Ahmed S, Stanley D, An C. Eicosanoid-mediated immunity in insects. Dev Comp Immunol. 2018;83:130–43.
13. Seo S, Lee S, Hong Y, Kim Y. Phospholipase A2 inhibitors synthesized by two entomopathogenic bacteria, Xenorhabdus nematophila and Photorhabdus temperata subsp. temperata. Appl Environ Microbiol. 2012;78:3816–23.
14. Ahmed S, Kim Y. Differential immunosuppression by inhibiting PLA2 affects virulence of Xenorhabdus hominickii and Photorhabdus temperata temperata. J Invertebr Pathol. 2018;157:126–46.
15. Hasan MA, Ahmed S, Mollah M, Lee D, Kim Y. Variation in pathogenicity of different strains of Xenorhabdus nematophila: differential immunosuppressive activities and secondary metabolite production. J Invertebr Pathol. 2019;166:107221.