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Youngjin Park
Andong National University

Yonggyun Kim
Andong National University, hosanna@andong.ac.kr

Hasan Tunaz
University of Nebraska-Lincoln

David W. Stanley
University of Nebraska-Lincoln

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An Entomopathogenic Bacterium, *Xenorhabdus nematophila*, Inhibits Hemocytic Phospholipase A2 (PLA2) in Tobacco Hornworms, *Manduca sexta*

Youngjin Park,[1] Yonggyun Kim,[1] Hasan Tunaz,[2] and David W. Stanley[2]

1. School of Bioresource Sciences, Andong National University, Andong 760-749, Republic of Korea
2. Insect Biochemical Physiology Laboratory, Department of Entomology, University of Nebraska, Lincoln, NE 68583-0816, USA

Corresponding author – Yonggyum Kim, email hosanna@andong.ac.kr

Abstract

The entomopathogenic bacterium, *Xenorhabdus nematophila*, induces immunodepression in target insects and finally leads to lethal septicemia of the infected hosts. A hypothesis has been raised that the bacteria inhibit eicosanoid-biosynthesis pathway to interrupt immune signaling of the infected hosts. Here, we show direct evidence that *X. nematophila* inhibits the activity of phospholipase A2 (PLA2), the initial step in the eicosanoid-biosynthesis pathway. Inhibition of PLA2 was dependent on both incubation time with *X. nematophila* and the bacterial concentration in in vitro PLA2 preparations of *Manduca sexta* hemocytes. While living bacteria inhibited PLA2 activity, heat-killed *X. nematophila* rather increased PLA2 activity. *X. nematophila* secreted PLA2 inhibitor(s), which were detected in the organic, but not aqueous, extract of the bacterial culture medium. The PLA2 inhibitory activity of the organic extract was lost after heat treatment. These results clearly indicate that *X. nematophila* inhibits PLA2 activity and thereby inhibits eicosanoid biosynthesis, which leads to immunodepression of the infected hosts.

Keywords: entomopathogenic bacterium, *Xenorhabdus nematophila*, *Steinernema carpocapsae*, *Manduca sexta*, phospholipase A2, immunodepression, hemocyte

1. Introduction

The gram-negative enterobacterium *Xenorhabdus nematophila* is an insect pathogen living in an entomopathogenic nematode, *Steinernema carpocapsae* (Thomas and Poinar, 1979). The infective juveniles of the nematode can enter target insect hosts through natural openings (spiracles, mouth, and anus). The nematodes penetrate into the insect hemocoel, where they release the symbiotic bacteria from their gut (Kaya and Gaugler, 1993; Dowds and Peters, 2002). The released bacteria induce immunodepression of their insect hosts to protect themselves and the nematodes from insect cellular and humoral immune reactions (Dunphy and Webster, 1984, 1991; Ribeiro et al., 1999). The bacteria kill their host insects within 16 h after being released into the hemolymph (Park and Kim, 2000). The insect cadavers become favorable monoxenic environments for nematode development and reproduction by the actions of antibiotics and hydrolytic enzymes secreted by the bacteria (Forst et al., 1997; Li et al., 1998). Newly hatched juvenile nematodes become infective by acquiring *X. nematophila* during development (Dowds and Peters, 2002). Upon reaching their dauer larval stage, the young nematodes leave their depleted hosts in search of new hosts, in which they complete the excursion of their life cycles.

The complex bacterium-nematode relationship is vulnerable to disruption by insect immune reactions to invasion and subsequent bacterial infection. It has been suggested, however, that *X. nematophila* protects the nematode life cycle by secreting active immunodepressive agents into newly infected insect hosts (Forst and Nealson, 1996). On the idea that prostaglandins and other eicosanoids are crucial mediators of insect cellular defense reactions, particularly nodulation reactions, to xenobiotic challenge (Miller et al., 1994; Stanley, 2000, 2002), Park and Kim (2000) proposed the hypothesis that one or more of the immunodepressive compounds secreted by *X. nematophila* acts by inhibiting eicosanoid biosynthesis. Their hypothesis was supported with results of a series of experiments which showed that larvae of beet armyworm, *Spodoptera exigua*, were impaired in their ability to develop melanized nodules following *X. nematophila* infection (Park and Kim, 2000). More to the point, treating the infected *S. exigua* larvae with arachidonic acid (AA; 20:4n-6), the main precursor to eicosanoid biosynthesis, reversed the nodule-forming impairment. Park et al. (2003) extended this idea in a similar line of work using tobacco hornworm, *Manduca sexta*.

Eicosanoids are formed by enzymatic oxygenation of AA or two other C20 polyunsaturated fatty acids, which are generally associated with the *sn*-2 position of cellular phospholipid (PL) pools (Stanley, 2000). The first step in eicosanoid biosynthesis is hydrolysis of AA from cellular PL pools, catalyzed by action of cellular phospholipase A2 (cPLA2; Baksinde et al., 1999; Dennis, 1997; Stanley, 2000). cPLA2s which are able to hydrolyze AA from the *sn*-2 position of PLs are present in tobacco hornworm fat body (Uscian and Stanley-Samuelsson, 1993) and hemocytes (Schleusener and Stanley-Samuelsson, 1996). The hemocyte cPLA2 is characterized by a marked preference for AA-linked PL substrate (Schleusener and Stanley-Samuelsson, 1996). More recently, we reported that bacterial infection stimulates increased PLA2 activity in tobacco hornworm hemocyte preparations (Tunaz et al., 2003). Based on this information we formulated the hypothesis that the immune impairing action of *X. nematophila* is due, in part, to inhibition of hemocyte PLA2

activity. Although several previous papers support the hypothesis by assessing PLA2 activity indirectly using eicosanoid biosynthesis inhibitors (Park and Kim, 2000) or by measuring a linked enzyme, phenoloxidase, activity (Park and Kim, 2003), no direct evidence has not been reported. Here, we report on the outcomes of experiments designed to test this hypothesis by directly measuring hemocyte PLA2 activity in response to *X. nematophila*. We also present the effects of incubation time and dose of the bacteria on hemocyte PLA2 activity for understanding pathogenic process during infection.

2. Materials and methods

2.1. Bacteria and insects

The bacterium, *X. nematophila*, was isolated from the hemolymph of the fifth instar larvae of *S. exigua* infected with *Steinernema carpocapsae* collected in Pochon, Korea (Park et al., 1999). The bacteria were cultured in tryptic soy broth (TSB, Difco, USA) at 28°C for 48 h with 150 rpm. Similarly, *Escherichia coli* K12 (a gift of Dr. R. Hutkins, UNL Department of Food Science and Technology) was cultured in TSB at 28°C for 48 h until stationary phase and suspended in MSB without medium. Tobacco hornworm, *M. sexta*, eggs were purchased from Carolina Biological Supply (Burlington, North Carolina). The larvae were reared on artificial diet under semi-sterile conditions (Stanley-Samuelson and Ogg, 1994). Second- and third-day fifth instars were used in all experiments.

2.2. Chemicals

Radiolabeled phosphatidylcholine (PC: 1-palmitol, 2-arachidonyl [arachidonyl-1-^{14}C], 1.9 GBq/mmol) was purchased from New England Nuclear (DuPont, Wilmington, Delaware). Analytical grade organic solvents were purchased from Fisher (Fair Lawn, New Jersey). Materials for *Manduca* saline buffer (MSB: 1.7mM Pipes {piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]}, 4mM NaCl, 40mM KCl, 18mM $MgCl_2$, 3mM $CaCl_2$, 243mM sucrose, 15 mg/L polyvinylpyrrolidone, pH 6.5), arachidonic acid (20:4n-6), ethylene glycol, and bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Sigma Chemical (St. Louis, Missouri).

2.3. Hemocyte PLA2 preparations

Hemolymph was collected by the pericardial puncture technique originally described by Dunn and Drake (1983). Hemocyte pellets were formed by centrifugation at approximately 1300g for 15 min at 4°C. The hemocytes were rinsed in MSB and repelleted three times. After the final rinse, the pellets were transferred to fresh 1.5 ml Eppendorf microcentrifuge tubes. Hemocytes were homogenized by sonication with eight 0.5 s bursts at 60W using a VibraCell sonicator (VibraCell, Danbury, Connecticut). The tubes were centrifuged at 1300g for 10 min at 4°C, and the supernatants were recentrifuged at 11,750g for 10 min at 4°C. Final supernatants were used to determine PLA2 activity. To standardize the PLA2 activity, protein concentrations of the reactive PLA2 preparations were determined using the bicinchoninic acid reagent (Pierce, Rockford, Illinois) against bovine serum albumin as quantitative standard at 562 nm using a BioTek microtiter plate reader.

2.4. PLA2 activity assay

Determination of PLA2 activities followed our routine protocols (Nor Aliza et al., 1999; Schleusener and Stanley-Samuelsson, 1996; Tunaz et al., 2003) in which release of radiolabeled arachidonic acid from phosphatidylcholine (1-palmitol, 2-arachidonyl [arachidonyl-1-^{14}C]) substrate was monitored by TLC.

2.5. The influence of **X. nematophila** *on hemocyte PLA2 activity: time course*

Hemocyte PLA2 preparations were preincubated for selected times in the presence of *X. nematophila* suspensions (10 μl from 10^8 cfu/ml). Control preparations were treated with MSB. After 1, 15, 30, 60, and 120 min incubations at room temperature, PLA2 activity was assessed in the preincubated mixtures as just described.

2.6. The influence of **X. nematophila** *on hemocyte PLA2 activity: bacterial dose-response*

Hemocyte PLA2 preparations were preincubated in the presence of bacterial suspensions at 10^2, 10^4, 10^6, or 10^8 cfu/ml. After 60 min preincubations, PLA2 activity was assessed as described.

2.7. The influence of heat-killed **X. nematophila** *on PLA2 activity*

A live *X. nematophila* suspension (108 cfu/ml) was heat-treated at 98°C for 30 min in a shaking water bath. The kill treatment was confirmed by plating 100 μl aliquots of the *X. nematophila* suspension on nutrient bromothymol agar plates (nutrient broth containing 0.0025% bromothymol blue and 0.004% 2,3,5-triphenyltetrazolium) and incubating the plates for 48 h. No bacterial colonies were observed on these plates.

Hemocyte PLA2 preparations were preincubated in the presence of heat-killed bacterial suspensions (10 μl). After 60 min preincubation, PLA2 activity was assessed as described. For positive controls, hemocyte PLA2 preparations were similarly preincubated with the same dose of living bacteria. To control for the possibility that nonpathogenic bacterial species may inhibit hemocytic PLA2 activity, hemocyte PLA2 preparations were preincubated with 10 μl of the *E. coli* K12 suspension (1×10^8 cfu/ml) for 60 min at room temperature. PLA2 activity was determined with four replications.

For negative controls, PLA2 preparations were preincubated in the presence of MSB (10 μl). PLA2 activity was assessed in the control preparations as described.

2.8. The influence of the organic fraction prepared from **X. nematophila** *culture medium on PLA2 activity*

Xenorhabdus nematophila was cultured in 200 ml TSB for 48 h at 28°C with 150 rpm. The culture medium was mixed with 200 ml ethyl acetate in an 1 L separatory funnel, and the phases were separated by centrifugation at 1250g for 30 min. The organic layer was collected and the ethyl acetate removed on a rotary evaporator fraction (Eyela, Japan) at 30°C. The organic extract was taken up in 1 ml of 50% ethanol.

To determine the effect of the organic fraction on the inhibition of the hemocyte PLA2 activity, 20 μl of the organic extract was added to the hemocyte PLA2 extract and preincubated for different times (1, 5, 10, 30, and 60 min) at room temperature. Controls were

treated with the same volume of MSB. PLA2 activity was measured as described above with four replications at each time point.

The influence of heat-treating the organic fraction on its ability to inhibit PLA2 activity was assessed. The organic fraction was heated for 30 min at 98°C in a shaking water bath. Hemocyte PLA2 preparations were preincubated at room temperature for 30 min with 20 ml of either heat-treated organic fraction or with 20 ml of untreated organic fraction. PLA2 activity was assessed as described.

2.9. Data analysis

Means and variance of the treatments were analyzed by one-way ANOVA by PROC GLM of SAS program (SAS Institute Inc., 1989). All means were compared by least squared difference (LSD) test at Type I error = 0.05.

3. Results

3.1. The influence of **X. nematophila** *on hemocyte PLA2 activity: time course*

Tobacco hornworm hemocyte preparations express substantial levels of PLA2 activity. Exposing the preparations to *X. nematophila* influenced hemocyte PLA2 activity (fig. 1). Hemocyte preparations exposed to living *X. nematophila* for 1 min expressed a significant increase in PLA2 activity, from about 0.8 pmol AA hydrolysis/mg protein/h to about 1.3 pmol AA hydrolysis/mg protein/h. Longer exposures to the bacterium, however, resulted in sharply reduced PLA2 activity. Hemocyte preparations exposed to living *X. nematophila* for 30 min expressed slight declines in PLA2 activity and after 60 min preincubation PLA2 activity declined in a statistically significant way to approximately 0.3 pmol AA hydrolysis/mg protein/h. The longer preincubation period of 120 min did not result in further decreases in PLA2 activity.

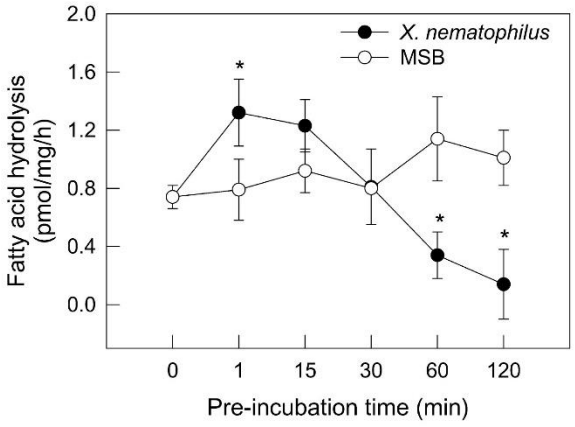

Figure 1. The influence of *X. nematophila* on the hemocyte PLA2 of *M. sexta* larvae: time course. The bacteria (10^6 cfu/10 μl) were preincubated with 250 μl of PLA2 extract (250 μg total proteins) in *Manduca* saline buffer (MSB) for the indicated times. Then the preincubated mixtures were added to radiolabeled phosphatidylcholine substrate and incubated

for 30 min at room temperature. PLA2 activity was calculated from hydrolysis of radioactive arachidonic acid. Each mean measurement consists of four replications. The asterisk above error bars indicates significant mean difference at Type I error = 0.05 (LSD test) between two treatments at each time point.

3.2. The influence of **X. nematophila** *on hemocyte PLA2 activity: bacterial dose-response*

The influence of *X. nematophila* on hemocyte PLA2 activity was expressed in a dose-dependent manner (fig. 2). Hemocyte PLA2 activity declined from about 1.2 pmol AA hydrolysis/mg protein/h after preincubations in the presence of 0 or 10 cfu of *X. nematophila* to approximately 0.8 pmol AA hydrolysis/mg protein/h in the presence of 10^2 cfu and to about 0.4 pmol AA hydrolysis/mg protein/h in the presence of 10^4 cfu. The higher dose of 10^6 cfu did not result in further declines in hemocyte PLA2 activity.

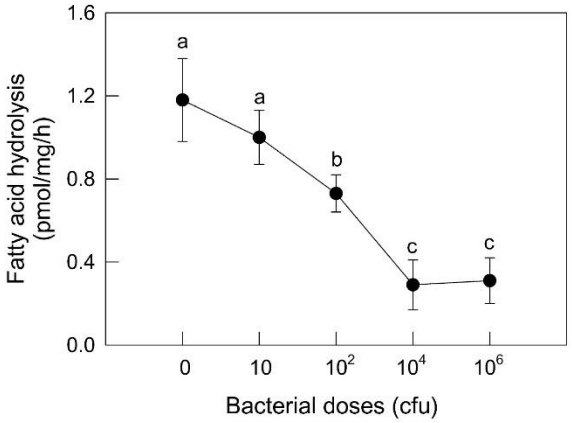

Figure 2. The influence of *X. nematophila* on the hemocyte PLA2 of *M. sexta* larvae: effect of dosage. Different concentrations of the bacteria (10 μl) were preincubated with 250 μl of PLA2 extract (250 μg total proteins) for 60 min. Then the preincubated mixtures were added to radiolabeled phosphatidylcholine substrate and incubated for 30 min at room temperature. PLA2 activity was calculated from hydrolysis of radioactive arachidonic acid. Each mean measurement consists of four replications. The different letters above error bars indicate significant mean difference at Type I error = 0.05 (LSD test).

3.3. The influence of heat-killed X. nematophila on PLA2 activity

Whereas exposing hemocyte preparations to living *X. nematophila* resulted in significant reductions in hemocyte PLA2 activity, similar exposure to heat-killed bacteria did not (fig. 3). To the contrary, hemocyte preparations preincubated with MSB or with live bacteria yielded PLA2 activity in the range of 1.0 pmol AA hydrolysis/mg protein/h and hemocyte preparations exposed to heat-killed *X. nematophila* expressed very high PLA2 activity, on the order of 7 pmol AA hydrolysis/mg protein/h. PLA2 reactions conducted in the presence of *E. coli* also stimulated substantial enzyme activity, similar to the results with heat-killed *X. nematophila* (fig. 3).

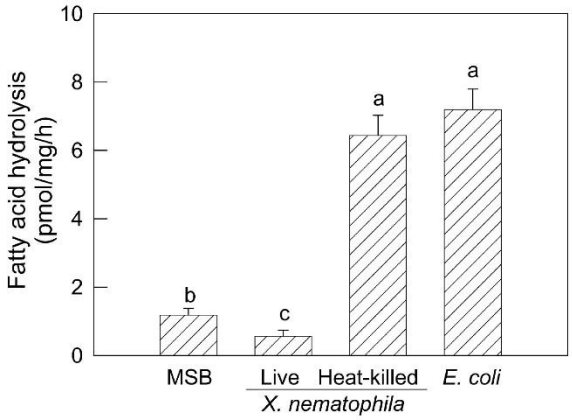

Figure 3. Influence of living and heat-killed *X. nematophila* and living *E. coli* K12 on PLA2 activity in hemocytes prepared from *M. sexta* larvae. Hemocyte PLA2 preparations were incubated in the presence of living *X. nematophila* and *E. coli*. For experiments with heat-killed *X. nematophila*, the bacteria were heated at 98°C for 30 min and preincubated with 250 ml of PLA2 extract (250 μg total proteins) in *Manduca* saline buffer (MSB) for 60 min. Then the preincubated mixtures were added to radiolabeled phosphatidylcholine substrate and incubated for 30 min at room temperature. PLA2 activity was calculated from hydrolysis of radioactive arachidonic acid. The histogram bars represent the mean of four replications and the error bars represent 1 standard error of the mean. Histogram bars annotated with the same letters are not significantly different at Type I error = 0.05 (LSD test).

3.4. The influence of the organic fraction prepared from **X. nematophila** *culture medium on PLA2 activity*

The organic fraction prepared from *X. nematophila* culture medium inhibited PLA2 activity in hemocyte preparations (fig. 4). Hemocyte preparations exposed to the organic extract for 30 and 60 min yielded severely reduced PLA2 activity (ca. 0.2 pmol AA hydrolysis/mg protein/h) compared to preparations that were not exposed to the organic fraction or were exposed to the fraction for relatively short preincubation periods (about 0.8 pmol AA hydrolysis/mg protein/h).

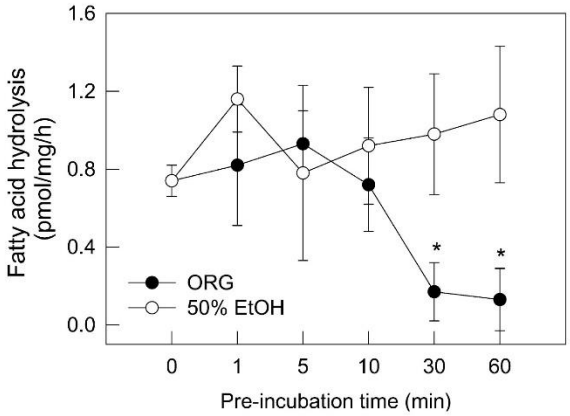

Figure 4. Time course of inhibitory effect of the organic fraction (ORG) extracted from the 48 h-cultured broth of *X. nematophila* on the hemocyte PLA2 activity of *M. sexta* larvae. The ORG (20 μl) was preincubated with 250 μl of PLA2 extract (250 μg total proteins) during the indicated times. Then the preincubated mixtures were added to radiolabeled phosphatidylcholine substrate and incubated for 30 min at room temperature. PLA2 activity was calculated from hydrolysis of radioactive arachidonic acid. Each mean measurement consists of four replications. The asterisk above error bars indicates significant mean difference at Type I error = 0.05 (LSD test) between two treatments at each time point.

The situation is otherwise for hemocyte preparations preincubated with heat-treated organic fractions (fig. 5). Hemocyte preparations incubated in the presence of 50% ethanol yielded about 2.1 pmol AA hydrolysis/mg protein/h, compared to approximately 0.25 pmol AA hydrolysis/mg protein/h for hemocyte preparations exposed to untreated organic fractions. Results for hemocyte preparations incubated in the presence of heat-treated organic fractions were statistically similar to the control ethanol treatments.

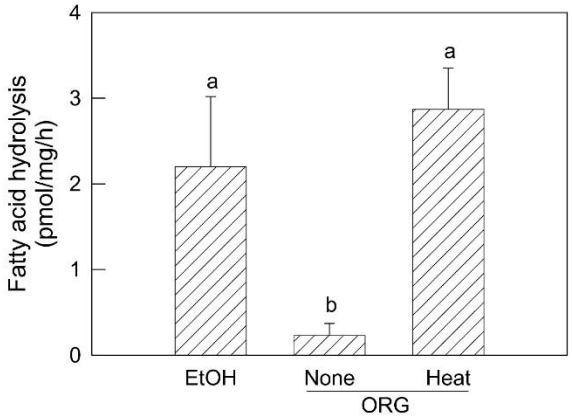

Figure 5. Heat treatment effect of the organic fraction (ORG) extracted from the 48 h-cultured broth of *X. nematophila* on the hemocyte PLA2 of *M. sexta* larvae. ORG was heated at 98°C for 30 min and preincubated with 250 μl of PLA2 extract (250 μg total proteins) in *Manduca* saline buffer (MSB) for 30 min. Then the preincubated mixtures were added to radiolabeled phosphatidylcholine substrate and incubated for 30 min at room temperature. PLA2 activity was calculated from hydrolysis of radioactive arachidonic acid. Each mean measurement consists of four replications. The different letters above error bars indicate significant mean difference at Type I error = 0.05 (LSD test).

4. Discussion

The results reported in this paper support our hypothesis that the bacterium, *X. nematophila*, impairs insect immune reactions to xenobiotic challenge by inhibiting the first step in eicosanoid biosynthesis, the PLA2 step. Several points support the hypothesis. First, preincubating hemocyte preparations with *X. nematophila* resulted in time-dependent inhibition of PLA2 activity, though there was a significant increase of PLA2 activity in 1 min preincubation time. The initial increase suggests that the hemocytes could recognize the bacteria and induce PLA2 activity, but after 30 min, the elevated PLA2 activity was reduced presumably by the material(s) released by *X. nematophila*. Second, the inhibitory influence of the bacterium was expressed in a dose-dependent manner. Third, the PLA2 inhibitory influence of the bacterium is destroyed by heat-kill treatments. The heat-killed bacteria rather increased the PLA2 activity. Similarly, reactions in the presence of a nonpathogenic bacterial species, *E. coli*, also stimulated high levels of enzyme activity. In the same type of assay, a gram-negative bacteria (freeze-dried), *Serratia marcescens*, also stimulated PLA2 activity (Tunaz et al., 2003). To show that the PLA2 inhibitory action of *X. nematophila* is a relatively unique and required pathogenic mechanism, it would be very informative to note that live form of nonpathogenic bacteria rather induce PLA2 activity, though several previous evidences strongly suggest the possibility (Jurenka et al., 1999; Yajima et al., 2003). Finally, a heat-labile factor or factors which appear in an organic fraction of the bacterial culture medium inhibit hemocyte PLA2 activity in a time-dependent manner. Taken together, we infer from these findings that *X. nematophila* produces and secretes one or more factors which somehow inhibit hemocytic PLA2 activity. Results of

our experiments with *S. marcescens* and *E. coli* add substantial support to this idea by indicating that secretion of PLA2-inhibitory factors is not a general feature of bacteria but may be restricted to *Xenorhabdus* and closely related bacterial genera.

In terms of the biological significance of our inference, it is recognized that the pathogenicity of *X. nematophila* depends on its ability to somehow impair the immune reactions of insect hosts (Dowds and Peters, 2002). Nonetheless, the mechanisms of impairing insect immunity and the kinetics of the impairment relative to the bacterial infection and colonization process have not been elucidated. Caldas et al. (2002) reported that *X. nematophila* produces and secretes a specific protease which eliminated nearly all induced cecropin activity in hemolymph of two insect larvae, *G. mellonella* and *Pseudaletia unipuncta*. Bacterial and other infections result in regulated activation of genes encoding cecropins and many other antibacterial proteins (Carlsson et al., 1991; Faye, 1990). However, antibacterial proteins do not appear in the hemolymph of infected insects until 6–12 h after infection. Due to the lengthy delay between the advent of an infection and the antibacterial protein reaction, the ability to inactivate cecropins or other such proteins would not provide *X. nematophila* much in the way of protection from the more immediate (and menacing) insect cellular defense reactions to bacterial challenge. The bacterium would require additional immune-impairing mechanisms.

Aside from its influence on antibacterial proteins, *X. nematophila* also acts to inhibit activation of prophenoloxidase in larval *G. mellonella*. While phenoloxidase is crucially important in immediate cellular defense and the longer-term humoral defenses (Gillespie et al., 1997), the defense actions of phenoloxidase occur in relation to other defense reactions. Beyond inhibiting phenoloxidase activation and from neutralizing antibacterial proteins, however, *X. nematophila* also exerts a directly cytotoxic effect on circulating hemocytes (Akhurst and Dunphy, 1993; Ribeiro et al., 1999). The cytotoxic effect would have long-lasting effects on the ability of an infected insect to defend itself from either an infecting bacterium or from the symbiotic nematodes associated with *X. nematophila*. Nonetheless, the cytotoxicity does not influence hemocyte population numbers in a way that would provide the invaders with immediate relief from insect immune reactions because it took more than several hours to reduce hemocyte population enough to block their immune reactions (Park and Kim, 2000).

Park and Kim (2000) hypothesized that *X. nematophila* inhibits nodulation reactions to infection and invasion. With respect to bacterial infection, nodulation is the earliest and quantitatively most prominent component of the insect cellular defense arsenal (Dunn and Drake, 1983). The implication of the Park and Kim hypothesis is that by inhibiting nodulation, *X. nematophila* is able to exert a rapid and catastrophically destructive influence on insect immune reactions. In this view, immediately after the bacterium is released from its nematode partner, *X. nematophila* disables the nodulation reaction. Attending this calamitous event, the ability of a host insect to recover its capability for nodulation some hours after infection is curtailed by killing the hemocytes required for nodulation. Most likely the insect will have died of overwhelming septicemia long before antibacterial proteins are produced. In the unlikely case these proteins do appear, however, they come out rather useless in the presence of bacterial proteases. The deadly virulence of *X. nematophila* is due, at least in part, to the multiple attacks on insect immune reactions.

A key element of the Park and Kim hypothesis lies in identification of a mechanism to account for impairing insect nodulation reactions. Park and Kim (2000) juxtaposed their hypothesis with the eicosanoid hypothesis put forth by Stanley and his colleagues. Eicosanoids are crucial mediators of nodulation and several other insect immune reactions to bacterial, fungal, and parasitoid challenge (Miller et al., 1994; Miller and Stanley, 2001; Stanley, 2000). It seems clear now the bacterium influences insect immunity by blocking the first biochemical step of eicosanoid biosynthesis in infected insects.

X. nematophila secretes a PLA2 inhibitory factor or factors which can be extracted into the organic phase of its culture medium. Because heat treatments with the bacterial cultures and with the organic fraction similarly destroy the PLA2- and immune-inhibiting actions, it seems likely the bacterial influence on insect immunity lies in the actions of these factors. We are not sure how these factors influence PLA2.

Because the experiments reported in this paper were performed with disrupted hemocytes over a rather short time course, it is unlikely the factors act by inhibiting biosynthesis of new PLA2 protein. Instead, they may act by direct competition with the active site of PLA2 protein or may act by interfering with the activation of the enzyme. Current effort is directed toward learning more about the factors.

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