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The Bacterium *Xenorhabdus nematophila* Inhibits Phospholipases A_2 from Insect, Prokaryote, and Vertebrate Sources

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Abstract
The bacterium *Xenorhabdus nematophila* is a virulent insect pathogen. Part of its pathogenicity is due to impairing cellular immunity by blocking biosynthesis of eicosanoids, the major recognized signal transduction system in insect cellular immunity. *X. nematophila* inhibits the first step in eicosanoid biosynthesis, phospholipase A_2 (PLA_2). Here we report that the bacterium inhibits PLA_2 from two insect immune tissues, hemocytes and fat body, as well as PLA_2s selected to represent a wide range of organisms, including prokaryotes, insects, reptiles, and mammals. Our finding on a bacterial inhibitor of PLA_2 activity contributes new insight into the chemical ecology of microbe-host interactions, which usually involve actions rather than inhibitors of PLA_2s.

Introduction

Insect innate immune systems are categorized into humoral (induced biosynthesis of antibacterial proteins) and cellular (direct interactions between invaders and circulating hemocytes) immune functions (Stanley 2004). While these systems provide effective protection from many invaders, some microbes are very potent insect pathogens because they are able to overcome or evade innate immune reactions. The Gram-negative enterobacterium *Xenorhabdus nematophila* is an intestinal symbiont of the insect pathogenic nematode *Steinernema carpocapsae*. *X. nematophila* enters insects with its host nematode, which defecates *X. nematophila* cells into the insect hemolymph, where they multiply and quickly kill

the insect. The freshly killed insect becomes an ideal microenvironment for nematode reproduction and development, from which a new generation of nematodes emerges in search of other hosts (Park and Kim 2000).

X. nematophila is an extremely virulent insect pathogen. Infections of less than 40 bacterial cells are lethal to tobacco hornworms, *Manduca sexta* (Forst et al. 1997) and beet armyworms, *Spodoptera exigua* (Park et al. 1999). Part of the virulence is due to bacterial factors which inhibit insect immune reactions to infection. *X. nematophila* is cytotoxic to insect hemocytes and inhibits prophenoloxidase activation (Dunphy and Webster 1991). More recently, Park and Kim (2000; Park et al. 2003) indicated that *X. nematophila* impairs cellular immune reactions by inhibiting biosynthesis of prostaglandins and other eicosanoids—signal moieties responsible for mobilizing insect cellular immunity (Stanley 2004).

Eicosanoid biosynthesis begins with action of a PLA_2, which hydrolyzes arachidonic acid from cellular phospholipids (Six and Dennis 2000). In insect systems, bacterial infection stimulates increased hemocytic PLA_2 activity (Tunaz et al. 2003). We recently learned that factors in *X. nematophila* culture medium directly inhibit insect hemocytic PLA_2 activity (Park et al. 2004). PLA_2s make up an enzyme superfamily of extraordinarily broad biological significance, including digestion, pathophysiology, phospholipid remodeling, cell signaling, and down-regulating cell signals (for example by hydrolyzing platelet-activating factor; Six and Dennis 2000). PLA_2s also act in organismal-level interactions, such as venom toxicity, microbial virulence, and host defense. Given the broad biological significance of these enzymes, microbes which produce PLA_2 inhibitors have potential to influence a wide range of PLA_2-mediated interspecific relationships. To consider this, we tested the idea that the *X. nematophila* factors responsible for inhibiting insect hemocyte PLA_2s also inhibit PLA_2s from other sources.

Materials and methods

Radioactive phosphatidylcholine [1-palmitol, 2-arachidonyl (arachidonyl-1-14C), 1.9 GBq/mmol] was purchased from DuPont New England Nuclear (Wilmington, Delaware). Analytical grade organic solvents were from Fisher (Fair Lawn, New Jersey). Arachidonic acid, EGTA and PLA_2s from *Streptomyces violaceoruber*, porcine pancreas, and from snake *Naja mossambica* and honey bee *Apis mellifera* venoms were purchased from Sigma (St. Louis, Missouri).

Tobacco hornworms, *Manduca sexta*, were reared from eggs (surface sterilized in 0.525% bleach) in individual, sealed 60-ml portion cups charged with sterilized culture medium (Park et al. 2003). Early fifth-stadium hornworms were used in all experiments.

The insect pathogenic bacterium, *Xenorhabdus nematophila* (strain K1), was cultured in tryptic soy agar (Difco, USA) at 28°C for 48 h. Bacteria were cultured in 25 ml of media in 50-ml flasks in a rotary shaker at 100 rpm and used in stationary phase. After washing the bacterial cells three times with sterile phosphate-buffered saline (10 mM phosphate buffer with 0.8% NaCl, pH 7.4), the cells were suspended in 1 ml of PBS as already described (Park et al. 1999). The suspended cells (10 μl; 10^8 cfu/ml) were used to assess the influence of bacterial cells on fat-body PLA_2 activity.

The *X. nematophila* PLA_2-inhibitory factors were prepared from overnight cultures (sufficient for inhibitory factor secretion) in the same conditions as described above. The culture medium was centrifuged for 30 min at 10,000 g. The cell-free supernatant was mixed with an equal volume of butanol, then separated into organic and aqueous fractions. The organic fractions from three extraction steps were combined and dried on a rotary evaporator at 40°C for 5 min. The organic extract was taken up in 1 ml of 50% EtOH; 10-µl aliquots were used to assess the influence of the organic extract on fat-body PLA_2 activity.

To fractionate the organic extract, the butanol was applied to a silica gel column (30 cm × 1 cm column, 70–230 mesh gel, Merck, Germany) and eluted in 20 steps (50 ml/step) with a linear concentration gradient of chloroform/methanol from 99:1 to 0:100 (v/v). Each fraction was dried, taken up in 1 ml of 50% ethanol, and 10-µl aliquots were assayed for the presence PLA_2-inhibitory activity. PLA_2-inhibitory activity was obtained in fractions 15 and 17 and these were designated ORG-1 and ORG-2 for the experiments reported here.

Determination of PLA_2 activities followed the protocols used by Tunaz et al. (2003) in which the release of radiolabeled arachidonic acid from phosphatidylcholine [1-palmitol, 2-arachidonyl (arachidonyl-1-^{14}C)] substrate was monitored by TLC. The PLA_2 assay was first optimized with respect to protein (100 mg optimal) and substrate concentrations (0.05 µCi optimal), reaction time (30 min optimal) and the optimal parameters were used in routine assays.

Results

Suspensions of *X. nematophila* and the organic extract prepared from the bacterial culture medium severely inhibited PLA_2 activity in tobacco hornworm fat-body preparations (see fig. 1; $F = 46.35$, $df = 2,6$, $P = 0.0002$). We recorded approximately 15 pmol/mg per min of PLA_2 activity in control preparations, which was reduced to approximately one-third that value in the presence of bacterial or organic extract preparations. After fractionating the organic extract into 20 fractions, we found that PLA_2 activity was severely curtailed in reactions conducted in the presence of ORG-1 and ORG-2. In addition to the inhibition of the fat-body PLA_2 activity, PLA_2 activity from four other sources was severely inhibited in reactions conducted in the presence of ORG-2 (see fig. 2; $F = 118.13$, $df = 7,16$, $P = 0.0001$). These PLA_2 sources represent secretory PLA_2s from a mammal (porcine pancreas), a reptile venom (snake, *N. mossambica*), an insect venom (honey bee, *A. mellifera*), and a prokaryote (bacterium, *S. vilaceoruber*).

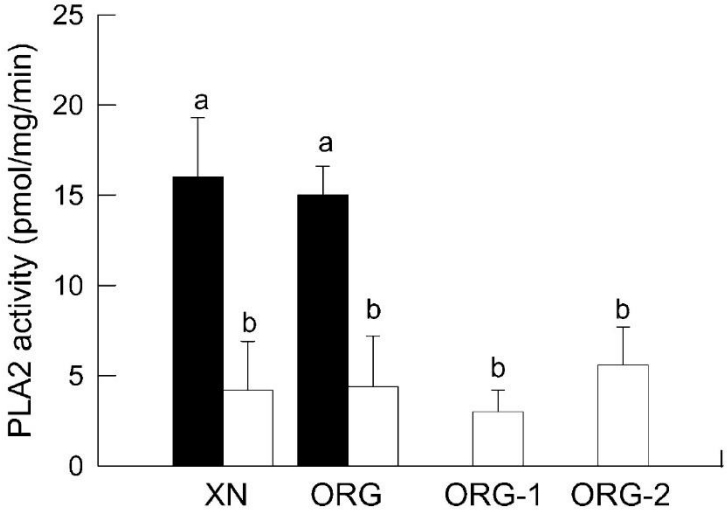

Figure 1. Inhibition of *M. sexta* fat-body PLA_2 activity in reactions carried out in the presence of *X. nematophila* (*XN*), or the organic extract of the bacterial culture medium (*ORG*), or two fractions prepared from ORG (*ORG-1* and *ORG-2*). Each histogram bar (solid fill for controls and unfilled for experimental treatments) displays mean PLA_2 activity (pmol/mg protein per min); the error bars represent 1 SD. Bars with the same superscript letter are not significantly different from each other (LSD, $P < 0.05$).

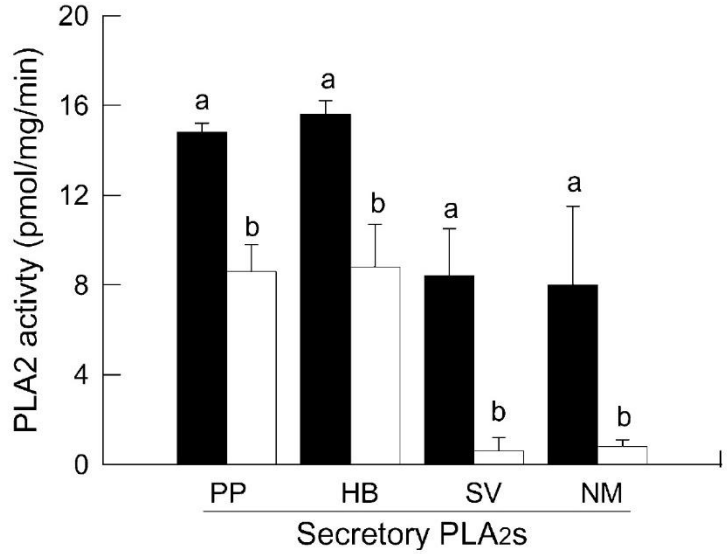

Figure 2. The influence of ORG-2 on PLA_2 activity of commercial enzymes prepared from porcine pancreas (*PP*), honey bee venom (*HB*), *Streptomyces vilaceoruber* (*SV*), and *Naja mossambica* (*NM*). Each histogram bar (solid fill for controls and unfilled for reactions conducted in the presence of ORG-2) displays mean PLA_2 activity (pmol/mg protein per min); the error bars represent 1 SD. Bars with the same superscript letter are not significantly different from each other (LSD, $P < 0.05$).

Discussion

The bacterium *X. nematophila* secretes PLA_2-inhibitory factors extractable from the culture medium into organic solvents. Our preliminary isolation work indicates the presence of two organic soluble factors. These factors inhibit the tobacco hornworm hemocytic PLA_2 (Park et al. 2004), the hornworm fat-body PLA_2, and secretory PLA_2s from porcine pancreas, the bacterium *S. vilaceoruber*, and venoms from the honey bee, *A. mellifera*, and the spitting cobra, *N. mosambica*. The *X. nematophila* factors are inhibitory to PLA_2s from a wide phylogenetic range of organisms, including prokaryotes, arthropods, reptiles, and insects.

The significance of this finding lies in the recognition that PLA_2s act in many organismal interactions. The primary function of the *X. nematophila* PLA_2-inhibitory factors seems to be impeding the biosynthesis of eicosanoids and thereby impairing insect cellular immune reactions to the presence of the bacterium. Impairment of host immune reactions may also be adaptive for the bacterium's symbiotic partner, the nematode, which could otherwise be isolated and killed by hemocytic encapsulation reactions. Beyond this, microbes produce and secrete many chemicals which act against other microbes. PLA_2s number among microbial products that can act against other microbes and also exert their toxic influences on hosts. Because the nematodes grow better in insect cadavers in which most of the living bacteria are *X. nematophila* (Forst et al. 1997), we suppose the *X. nematophila* PLA_2-inhibitory factors also serve in reducing the numbers of other microbes.

Part of the virulence of many microbes, including viruses (Canaan et al. 2004), fungi (Nakashima et al. 2003), and bacteria (Phillips et al. 2003), involves secretion of toxic microbial PLA_2s; however, the details of PLA_2-mediated microbe-host interactions can be complex. At least some toxic PLA_2s are thought to exert their influence via receptor-mediated events (Cupillard et al. 1999) and via direct cell damage. A new variation on this theme was recently discovered. A PLA_2 from the pathogenic bacterium *Pseudomonas aeruginosa* (the cytotoxin ExoU) requires the presence of host cell factors for activation (Phillips et al. 2003). Hence, the pathogenicity expressed via ExoU requires cooperation with the injured host. While microbial PLA_2s can be toxic, mammalian hosts also produce and secrete PLA_2s which are bactericidal via hydrolysis of the outer membrane of Gram-positive bacteria (Dubouix et al. 2003). We infer that a great deal of new information will emerge on PLA_2-mediated microbe-host interactions. The idea that a bacterial species produces and secretes PLA_2-inhibitory factors would be seen as still another variation on the theme.

Finally, we suspect that secretion of broad-spectrum PLA_2 inhibitors will not be restricted to representatives of other *Xenorhabdus* and closely related *Photorhabdus* species.

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