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Interaction of the bacteria *Xenorhabdus nematophila* (Enterobactericeae) and *Bacillus subtilis* (Bacillaceae) with the hemocytes of larval *Malacosoma disstria* (Insecta: Lepidoptera: Lasiocampidae)

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Abstract

Malacosoma disstria larvae are a pest of deciduous trees. Little is known on the interaction of bacteria with the immediate hemocytic antimicrobial responses of these insects. Incubating dead *Xenorhabdus nematophila* and *Bacillus subtilis* with a mixture of serum-free granular cells and plasmatocytes *in vitro* revealed differential bacterial-hemocyte adhesion and differential discharge of lysozyme and phenoloxidase but not total protein. Although active phenoloxidase adhered equally to both bacterial species, *X. nematophila* limited enzyme activation whereas *B. subtilis* enhanced activation. Serum with active phenoloxidase (as opposed to tropolone-inhibited phenoloxidase) and purified insect lysozyme increased bacterial-hemocyte adhesion of both bacterial species. An apolipophorin-III-like protein when incubated with hemocytes, limited their responses to glass slides and bacterial adhesion. However, initial binding of the protein to both bacteria increased granular cell levels with bacteria while lowering the plasmatocyte levels with adhering procaryotes. The protein also increased lysozyme and phenoloxidase activities. Although *B. subtilis in vivo* elicited a nodulation-based decline in total hemocyte counts and did not affect hemocyte viability, dead *X. nematophila* elevated hemocyte counts and damaged the hemocytes as lipopolysaccharide levels increased and *X. nematophila* emerged into the hemolymph. Apolipophorin-III-like protein once bound to the bacteria slowed their removal from the hemolymph.

Keywords: *Malacosoma*; *Xenorhabdus*; Hemocytes; Lysozyme; Phenoloxidase; Plasma protein

1. Introduction

There are few well-defined models of the hemocytic antimicrobial systems of the Lepidoptera, the salient examples being the economic pest insects *Manduca sexta* and *Galleria mellonella*, and beneficial *Bombyx mori* (Gillespie et al., 1997), all of which occupy different niches and have nuance differences in their antibacterial systems (Gillespie et al., 1997). *Malacosoma disstria* was chosen as a model because, unlike the previously considered insects, it is a major gregarious native pest of north american deciduous trees (Furniss and Carolin, 1997). Additionally, *M. disstria* hemolymph supports the growth and development of numerous biological control agents including parasitoids and micro-organisms (Stoltz and Guzo, 1986) as well as the nematode, *Steinernema carpocapsae*, and its mutualistic bacterium, *Xenorhabdus nematophila* (personal observation).

In general, the innate antimicrobial systems in lepidopteran hemolymph consist of interactive humoral and cellular factors (Gillespie et al., 1997). Salient humoral

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elements include the opsonic, melanizing phenoloxidase system (Lockey and Ourth, 1996), lysozyme (a cationic, constitutive plasma protein) (Lockey and Ourth, 1996; Wilson and Ratcliffe, 2000), C-type lectins, β-1,3-glucan-binding proteins, hemolin, peptidoglycan-binding proteins (see Yu and Kanost, 2002) and apolipophorin-III (ALP, Halwani et al., 2000), many of which facilitate the binding micro-organisms to hemocytes. ALP is a multifunctional plasma protein in *G. mellonella* that binds to lipoteichoic acids from avirulent *Bacillus subtilis* impairing phenoloxidase activation (Halwani et al., 2000), neutralizes toxic lipopolysaccharides (endotoxins) of virulent *X. nematophila* (Dunphy and Halwani, 1997), adheres to bacterial surfaces affecting hemocyte activity (Zakarian et al., 2002, 2003), activates the prophenoloxidase cascade in *G. mellonella* (Park et al., 2005), potentiates lysozyme activity against bacterial surfaces (Halwani and Dunphy, 1999) and is a β-1,3-glucan recognition protein (Dunphy et al., 2003; Whitten et al., 2004) enhancing encapsulation of yeast (Whitten et al., 2004). The cellular antibacterial components include the hemocyte types, the plasmatocytes and granular cells, which, depending on the insect species and bacterial species and concentration (Howard et al., 1998), participate in phagocytosis (Tojo et al., 2000) and nodule formation (Ratcliffe et al., 1985). Nodulation is a biphasic response in which micro-organisms adhere to granular cells producing a coagulum that is walled off by the plasmatocytes (Ratcliffe et al., 1985).

Little is known about the immediate innate hemocyte responses of *M. disstria* to foreign materials. Air-dried hemolymph smears of *M. disstria* provide a foundation hemogram (Arnold and Sohi, 1976). Polydnaviruses suppress phenoloxidase activity and phagocytosis of yeast (Stoltz and Guzo, 1986). As part of a non-self response, the biogenic amine, octopamine, elevates the secondary cellular messenger, cyclic AMP in a *M. disstria* hemocyte tissue culture (Gole et al., 1982). Protein kinase C and cyclic AMP-dependent protein kinase A influence the adhesion of fifth instar *M. disstria* granular cells and two plasmatocyte morphotypes to glass (Giannoulis et al., 2005). Herein, emphasis is placed on the immediate interaction of the insect hemocytes with the surfaces of dead *B. subtilis* and *X. nematophila*. Dead bacteria were used to define their surface factors participating in the antimicrobial hemocyte response without influence from bacterial metabolism. Consideration is given to the involvement of phenoloxidase, lysozyme and ALP, molecules known to affect lepidopteran hemocyte responses (Gillespie et al., 1997; Halwani et al., 2000).

Bacillus subtilis binds avidly to hemocytes of *G. mellonella* (Zakarian et al., 2002). Lipoteichoic acids of these bacteria elicit nodulation, deplete the plasmatocyte concentration in the hemocoel and irreversibly damage granular cells while activating phenoloxidase of larval *G. mellonella* (Halwani et al., 2000). Vectored into insect hemolymph by the entomopathogenic nematode, *S. carpocapsae* (Akhurst, 1980), *X. nematophila* limits phenoloxidase activation (Dunphy and Webster, 1988, 1991) possibly by chelating Ca^{2+} ions (Yokoo et al., 1992). This micro-organism impairs also hemocyte function by releasing iron-chelators from hemocytes damaged by bacterial lipopolysaccharides (Dunphy et al., 2002), while tolerating lysozyme-induced modification of the bacterial envelope (Dunphy and Webster, 1991). In *Spodoptera exigua*, live *X. nematophila* impairs eicosanoid biosynthesis (Park and Kim, 2000). Eicosanoids are part of the signal transduction system of insect hemocytes (Morishima, 1998) affecting hemocyte phagocytosis and spreading (Mandato et al., 1997).

Here, we determine that the extent of binding of *X. nematophila* and *B. subtilis* to *M. disstria* hemocytes varies with the bacterial species and hemocyte type with mediation by lysozyme and phenoloxidase and possibly phenoloxidase metabolites. ALP binds to the bacteria limiting the adhesion of both bacterial species to plasmatocytes and enhances the number of granular cells with either bacterial species. *B. subtilis* is removed from the hemolymph by nodulation more rapidly than *X. nematophila*, the removal of both bacterial species being slowed by ALP. Unlike the former bacterial species, *X. nematophila* subsequently emerges into the hemolymph as endotoxin levels increase and hemocyte viability declines independently of ALP bound to the bacteria.

2. Materials and methods

2.1. Bacteria

Stock cultures of the phase one form of *X. nematophila* ATCC19061 [the form released from the infective stage of the nematodes upon entering the insect hemolymph (Akhurst, 1980)] were grown on Luria agar supplemented with triphenyltetrazolium chloride (30 mg/L) and bromthymol blue (25 mg/L). *B. subtilis* (Boreal Biological Co.) was grown on Luria agar. Both bacterial species were incubated at 25 °C in darkness and subcultured biweekly.

For experimental purposes, bacteria were grown to the mid-exponential phase of the growth cycle (turbidity at 660 nm = 0.75) in 5 mL of Luria broth in 20 mL scintillation vials at 28 °C on a horizontal gyratory shaker (250 rpm). Bacteria were washed three times by centrifugation (12,000*g*, 2 min, 25 °C) and resuspension in 5 mL of phosphate-buffered saline (138 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2.8 mM KH_2PO_4, pH 6.5; PBS). The bacteria were killed by UV-irradiation for 3 h and were then stored overnight at 5 °C. Bacterial death was confirmed by (i) the absence of a change in turbidity of Luria broth inoculated with UV-irradiated bacteria and incubated for 96 h and (ii) no discernible colony formation when the bacteria were plated on Luria agar and incubated for 96 h at 28 °C. Cultures were centrifuge-washed in PBS prior to use. Dead bacteria precluded the effects of formyl peptides (Alavo and Dunphy, 2004) and other aspects of metabolism (Park and Kim, 2000) influencing results allowing the direct observation