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Effects of entomopathogenic bacterium *Photorhabdus temperata* infection on the intestinal microbiota of the sugarcane stalk borer *Diatraea saccharalis* (Lepidoptera: Crambidae)

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ABSTRACT

Photorhabdus temperata is an entomopathogenic bacterium that is associated with nematodes of the Heterorhabditidae family in a symbiotic relationship. This study investigated the effects of *P. temperata* infection on the intestinal microbiota of the sugarcane stalk borer *Diatraea saccharalis*. Histopathology of the infection was also investigated using scanning electron microscopy. Groups of 20 larvae were infected by injection of approximately 50 bacterial cells directly into the hemocoel. After different periods of infection, larvae were dissected and different tissues were used for bacterial cell quantification. *P. temperata* was highly virulent with an LD_{50} of 16.2 bacterial cells at 48 h post-infection. Infected larvae started dying as soon as 30 h post-infection with a LT_{50} value of 33.8 h (confidence limits 32.2–35.6) and an LT_{90} value of 44.8 h (CL 40.8–51.4). Following death of the larvae, bacteria from the midgut did not invade the hemocoel. In the midgut epithelium, *P. temperata* occupied the space underneath the basal lamina. The cultivable intestinal bacterial populations decreased as soon as 1 h post-infection and at 48 h post-infection, 90% of the gut microbiota had died. The role of *P. temperata* in control of the midgut microbiota was discussed.

1. Introduction

Sugarcane is one of the most important crops in Brazil, cultivated since the arrival of Europeans. Nowadays, as a result of the search for sustainable sources of fuels, interest in cultivation of sugarcane has revived. However a major pest of this crop, the moth *Diatraea saccharalis* (Lepidoptera: Crambidae), commonly known as the sugarcane stalk borer, is responsible for large losses in production (Huang et al., 2007). The adult moth lays eggs on the leaves and the 1st and 2nd larval instars feed on the leaves before boring into the plant stems. The use of chemicals pesticides to control *D. saccharalis* is not very efficient due to the cryptic nature of this pest, and causes a reduction of natural enemies populations. The use of Micro-hymenopteran parasitoids for biological control of this pest started in the 1980s and has become one of the most valuable biopesticides used currently in commercial sugarcane crop management in Brazil (Degaspari et al., 1987). Although *D. saccharalis* can be controlled by this method, there is still a need for further research on new approaches to pest management (Falco et al., 2001).

Terra and Ferreira (2005) state that the insect midgut is one of the most important interfaces between the animal and its environment and in the case of plant feeding insects, the midgut epithelium is directly challenged by plant defensive compounds. Anther important component of the midgut physiology is related to the gut microbiota. Recent studies have demonstrated that many insects are dependent on the gut microbiota for detoxification of harmful substances or for the supply of essential nutrients among other functions (Dillon and Dillon, 2004). The search for compounds that could act specifically and adversely on the crucial bacterial species associated with insect pests is required.

Promising sources of such compounds are the entomopathogenic bacteria of the genus *Photorhabdus* and *Xenorhabdus*. These bacteria live in a symbiotic association with nematodes of the families Heterorhabtidae and Steinernematidae, respectively (Thomas and Poinar, 1979; Bowen et al., 1998). The bacteria, when released by the nematodes into the hemolymph of the host insect, produce a mixture of toxins that not only result in host death, but also inhibit potential competitors, such as other bacteria, nematodes, fungi and even vertebrates predators/scavengers (Hu and Webster,

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2000; Issacson and Webster, 2001; Acevedo et al., 2007; ffrench-Constant et al., 2007).

During the early events of the *Photorhabdus luminescens* infection in larval *Manduca sexta*, toxins act on the immune defenses of the insect and a sub-population begins growing in a highly restricted region between the basal lamina and the cells of the midgut epithelium (Daborn et al., 2002; Silva et al., 2002; Au et al., 2004). It is still not clear why *P. luminescens* initiate their colonization associated with the proximal region of *M. sexta* midgut. What it is known is that at very early stages of the infection, when the number of the invading bacteria is still very low, they have to confront the cellular and humoral defenses of their hosts. In that case, the secretion of potent toxins, which act directly against the hemocytes or host enzymes are adaptive (Eleftherianos et al., 2007). It is possible that the subpopulation that migrates to the midgut tissue is another front of the attack carried out by *Photorhabdus*, whose function is to control the main source of immediate competitors, the midgut luminal microbiota.

The study described in the present paper was undertaken to shed light on the role of the sub-population of *Photorhabdus temperata*, a species closely related to *P. luminescens*, which is also capable of invading the same niche between the basal lamina and the midgut cells of the host. We were interested to investigate how the *Photorhabdus* infection may affect *D. saccharalis* midgut microbiota.

2. Materials and methods

2.1. Rearing of insects

Sugarcane stalk borer larvae were reared on artificial diet as described by Hensley and Hammond (1968). The incubation room was maintained at 28 °C and 60% relative humidity with a natural photoperiod.

2.2. Pathogenicity tests

The bacterial strain used in this study was *P. temperata* strain K122, kindly supplied by Prof. Richard ffrench-Constant (University of Bath, UK). Primary variant colonies were inoculated into sterile tubes containing 2 mL of Luria Bertani broth. The cultures were incubated for 24 h at 30 °C on a rotating shaker at 340 rpm. For insect injection, bacterial cells were washed with sterile phosphate-buffered saline (PBS; 0.15 M sodium chloride, 10 mM sodium phosphate buffer, pH 7.4) and resuspended in this same buffer. Fourth-instar *D. saccharalis* larvae were injected with 10 μL of a suspension containing washed bacterial cells. Injections were performed directly into the insect hemocoel using a 50-μL Hamilton syringe with a 30-gauge needle. The number of injected bacteria was confirmed by plating a known volume of the injected suspension onto Plate Count Agar. Post-injection, larvae were maintained individually on artificial diet, and symptoms of pathogenicity were noted overtime.

2.3. Bacterial colonization

To document the number of recoverable bacteria within different infected tissues over time, replicates of five larvae were dissected at different time points after injection. Each larva was surface sterilized with 70% ethanol and then bled to collect the maximum volume of hemolymph possible. After bleeding, each larva was dissected to separate the internal organs. The midgut and fat body were individually homogenized in PBS using a hand held Potter–Elvehjem homogenizer. The rest of the insect body was defined as the 'carcass', and homogenized as described above. To determine the number of recoverable bacteria (CFU per tissue or hemolymph), serial dilutions of tissue homogenates and hemolymph samples were prepared with PBS and plated onto Plate Count Agar and Mackonky Agar. Cultures were incubated for 48 h at 30 °C and individual colonies counted.

2.4. Scanning electron microscopy

For scanning electron microscopy (SEM), larvae were dissected in PBS to expose internal organs. Midguts were then fixed in 2.5% glutaraldehyde in PBS, post-fixed in 1% osmium tetroxide, dehydrated through an acetone series, and critical point dried. Samples were then coated with gold and examined using a Zeiss SEM 962 scanning electron microscope.

3. Results

3.1. Pathogenicity of P. temperata strain K122 against larval D. saccharalis

Photorhabdus temperata strain K122 was highly virulent when tested against fourth instar larvae of *D. saccharalis* with an LD_{50} of 16.2 bacterial cells (confidence limits 10.1–22.5) calculated by Probit analysis at 48 h post-infection. An *E. coli* strain used as a negative control, and showed no pathogenicity at a concentration of 10^8 cells (data not shown). We chose to use 50 bacterial cells for subsequent infections of *D. saccharalis* larvae. Infected larvae started dying as soon as 30 h post-infection with a LT_{50} value of 33.8 h (confidence limits 32.2–35.6) and an LT_{90} value of 44.8 h (CL 40.8–51.4). The majority died between 48 and 60 h after infection and at this time the larvae turned a red-brown color typical of insects infected with *P. temperata*.

Following death of the larvae and subsequent rupture of the degenerating midgut epithelium, bacteria from the intestinal microbiota did not invade the hemocoel. Only *P. temperata* colonies were observed after plating of hemolymph and other internal organs from infected larvae (data not shown).

3.2. Colonization of different insect tissues by P. temperata

We examined the relative growth of *P. temperata* in different insect compartments by measuring the number of recoverable colony-forming units (CFU) per injected larva over time. After injection of 50 bacterial cells, the number of bacteria recoverable from either the hemolymph or the midgut epithelium increased steadily overtime (Fig. 1). The number of recoverable *P. temperata* cells increased by six orders of magnitude within 48 h (24–72 h post-infection). There was a delay in colonization of, and multiplication within, the rest of the insect body, here referred to as the carcass (Fig. 1).

In the midgut epithelium, scanning electron microscopy showed that *P. temperata* occupied the space between the basal membrane of epithelial cells and the basal lamina that surrounds the midgut (Fig. 2). Once beneath the basal lamina, the bacteria are closely associated with the cells of midgut epithelium.

3.3. Effects of the midgut tissue colonization on the intestinal microbiota

To document if cells of *P. temperata* within the epithelium-basal lamina niche produce antibacterial factors, we investigated the viability of the cultivable intestinal microbiota at different periods after infection. Fig. 3 shows that the number of cultivable intestinal bacteria decreased overtime following infection. The number of cultivable intestinal bacteria started to decrease as