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Short communication

Direct infection of *Spodoptera litura* by *Photorhabdus luminescens* encapsulated in alginate beads

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Abstract

Actively growing cultures of *Photorhabdus luminescens* were encapsulated in sodium alginate beads and examined for their ability to infect insect hosts. These beads, containing approximately 2.5×10^7 *Photorhabdus* cells per bead, when mixed with sterilized soil and exposed to *Spodoptera litura* larvae resulted in 100% mortality in 48 h, while the use of alginate encapsulated *Heterorhabditis* nematode resulted in 40% mortality after 72 h. The bacteria were reisolated from the dead insect thus proving Koch's postulates and demonstrating the ability of *P. luminescens* to kill the insect host on their own, independent of the symbiont nematode. The LC_{50} dose of *Photorhabdus* cells was estimated at 1010 cells per larva for killing *S. litura* 6th instar larvae in 48 h.

Keywords: Pathogenicity; Insect; *Heterorhabditis*; *Photorhabdus*; Nematode; Alginate beads

1. Introduction

The motile Gram-negative bacteria *Photorhabdus luminescens*, potent insect pathogens, exist as symbionts of the entomopathogenic nematodes *Heterorhabditis* spp. (Boemare et al., 1993; Kaya and Gaugler, 1993). The inability to isolate these bacteria in a free-living form from the environment gave raise to doubts about their capacity to survive, multiply and infect insects in soil independent of their symbiont nematodes (Forst and Nealson, 1996). Studies by Gotz et al. (1981) showing that the nematode helps in overcoming the host's (insect's) defense by secreting an immune inhibitor served to reinforce these doubts. Nevertheless efforts to establish the insect pathogenicity of *P. luminescens* in isolation from the nematode host continued. To check out the independent viability of the bacteria in soil, Poinar et al. (1980) investigated the persistence of *Photorhabdus* in sterile soil and were unable to recover any cells even on the day following the inoculation of the soil with the bacteria. However, Bleakley and Chen (1999) reported the successful survival of these bacteria for up to one month, in sterile (autoclaved) acidic soil augmented with calcium carbonate and gelatin or casamino acids, but they did not evaluate the insect pathogenicity of *Photorhabdus* under these conditions.

All reports, until date, examining the direct virulence of *P. luminescens* to insect larvae employed artificial means, such as injecting live bacterial cells into the larvae (Bowen and Ensign, 1998; Gotz et al., 1981; Rajagopal and Bhatnagar, 2002). In an attempt to effect a convergence of the directions charted out by all the earlier studies, we have focused on investigating the ability of *P. luminescens* in the soil environment to independently infect and kill an insect larvae. As such, we encapsulated *P. luminescens akhurstii* in sodium alginate beads for release in soil to assess their ability to kill *Spodoptera litura* 6th instar larvae upon feeding.

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2. Materials and methods

2.1. Culturing of bacteria and alginate beads preparation

Photorhabdus luminescens sub sp. *akhurstii* strain K-1, isolated from *Heterorhabditis indica*, and *Escherichia coli* strain K-12 were grown overnight on Luria Bertani (LB) medium from starter cultures at 0.5% inoculum concentration. They were then processed as follows to result in six different alginate bead preparations.

T1 *P. luminescens K-1*; overnight cell suspension (50 ml) containing approximately 1×10^9 cells/ml; medium was used without centrifugation to form beads in the ratio of 2.5×10^7 *P. luminescens* cells per bead.

T2 *P. luminescens K-1*; overnight cell suspension centrifuged at 15,000*g* at 4 °C, pellets resuspended in fresh LB medium (50 ml) containing approximately 1×10^9 cells/ml; beads formed in the ratio of 2.5×10^7 *P. luminescens* cells per bead.

T3 *P. luminescens K-1*; supernatant from T2 passed through 0.22 μm filter (50 ml); beads formed in the ratio of 0 *P. luminescens* cells per bead.

T4 *E. coli K-12*; overnight cell suspension (50 ml) containing 1×10^9 cells/ml; medium was used without centrifugation to form beads in the ratio of 2.5×10^7 *E. coli* cells per bead.

T5 *Sterile water* (50 ml); beads formed in the ratio of 0 *P. luminescens* cells per bead.

T6 *H. indica* infective juveniles (IJ) in 50 ml sterile water; contained approximately 300 IJ/ml; beads formed in the ratio of 7.5 *H. indica* IJ's per bead.

Each 50 ml treatment was mixed thoroughly with 50 ml of a solution containing 2% sodium alginate and 2% sucrose in sterile water and was then introduced dropwise into a 1.47% calcium chloride solution resulting in the formation of uniformly round 3–4 mm diameter beads. After 30 min, the calcium chloride was decanted and the beads were collected on a wire mesh filter. They were washed/flushed five times in succession with approximately 200 ml of sterile water each time, and were allowed to decant fully. The resulting beads were used immediately or were stored at 28 °C in polythene bags. Further, serial dilutions were made of the pelleted and resuspended *P. luminescens* preparation (T2) resulting in beads having a range of cells—from 2.5×10^7 cells per bead to 2.5 cells per bead.

2.2. Insect bioassays

Spodoptera litura were reared according to Rajagopal and Bhatnagar (2002). A 60 mm diameter Petri dish (Falcon) was half-filled with sterile soil and four alginate beads were distributed in it. One 6th instar *S. litura* larvae were released into each Petri dish and were observed until 48 h for mortality. For each of the above treatments there were 10 replications and each replication consisted of five Petri plates having one insect each. All the bioassay experiments were conducted at room temperature (28 °C). The mortality data were recorded at 48 h post exposure of the insects to the different treatments.

3. Results and discussion

Alginate beads prepared from the six different treatments were tested for insect pathogenicity on *S. litura* larvae. All the insects tested with alginate beads containing *P. luminescens* cells (T1, prepared from *P. luminescens* broth and T2, prepared by pelleting and resuspending the *P. luminescens* cells) resulted in 100% mortality within 48 h, while there was no mortality in the insects tested with alginate beads of *E. coli* and sterile water. The dead insects turned reddish in colour and luminesced strongly in dark. The bacteria were isolated from the body of the dead insect, the genomic DNA prepared and a PCR–RFLP analysis was conducted on the 16S rDNA gene according to Rajagopal and Bhatnagar (2002). The PCR–RFLP profile of the reisolated bacteria and the initial bacteria (K-1) was identical thus satisfying all Koch's postulates and establishing the fidelity of infection and pathogenicity. *Photorhabdus* secretes insecticidal proteins into the culture medium, which have been purified (Bowen and Ensign, 1998; Rajagopal and Bhatnagar, 2002) and their encoding genes cloned (Bowen et al., 1998). Alginate beads prepared from the culture supernatant (T3) were also fed to the larvae to discriminate between the larval deaths due to the secreted toxin as against the direct infection of the insect by *P. luminescens* cells. Exposing the larvae to these alginate beads resulted in 20% mortality within 48 h, which pointed to retention of the secreted toxin protein complex's insecticidal activity in the alginate beads.

To understand the progress and mortality of infection, the larval behaviour every six hours after releasing them on alginate beads containing *Photorhabdus* cells (T2) was monitored. The insects could be seen moving around and nibbling at the beads at the 6th hour, and by the 12th hour approximately 40% of the beads were consumed and the larval mobility appeared significantly reduced. By the 18th hour the larvae were immobile, and by the 24th hour they were dead. They turned reddish-black in colour and luminesced strongly in dark by the 48th hour. At each of the above four intervals, soil samples were taken and analysed for the presence of *Photorhabdus* colonies by serial dilution. No *Photorhabdus* CFU could be identified.

Heterorhabditis indica IJ (carrying *Photorhabdus* in their gut) encapsulated in alginate beads (T6) did not result in the mortality of *S. litura* larvae until 48 h. (Mortality from the nematode treatment started to occur only after 72 h.) A comparison of the cadavers of the larvae killed by *P. luminescens* alone to those killed by *H. indica* (Fig. 1) revealed that while in the former there is complete degradation of the larval cadaver, in the latter the integrity of the cadaver was retained. A plausible explanation for this could be that as *Heterorhabditis* are bacterial feeders they need a constant supply of bacteria for completing their life cycle.