Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus–Heterorhabditis* infected *Galleria mellonella* larvae

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Abstract

The population of *Photorhabdus luminescens* C9, bacterial symbiont of the entomopathogenic nematode, *Heterorhabditis megidis* 90, increased rapidly to 1.2–2.6 × 10⁹ cells g⁻¹ wet *Galleria mellonella* larvae within 24 h of nematode infection of the larvae, and maintained a relatively constant level (1.2–2.0 × 10¹⁰ cells g⁻¹) through the entire 14-day period of nematode development. The antibiotic, 3,5-dihydroxy-4-isopropylstilbene, was produced by *P. luminescens* C9 after 24 h of nematode infection, increased rapidly at 2–5 days postinfection and remained at a level of 3000–3600 W g⁻¹ wet larvae until about 21 days, decreasing gradually thereafter. The early production and continued presence of a relatively large amount of 3,5-dihydroxy-4-isopropylstilbene in the infected insect supports the hypothesis that the antibiotics produced by the bacterial symbiont help minimize competition from other microorganisms and prevents the putrefaction of the nematode-infected insect cadaver. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Photorhabdus* spp. (Enterobacteriaceae) are bacterial symbionts of soil-living entomopathogenic nematodes, *Heterorhabditis* spp. (Nematoda: Heterorhabditidae) except for *Photorhabdus asymbiotica*, which was reported in clinical samples of human wounds [1]. The infective juveniles (IJs) of the nematode carry the bacterial symbiont in their intestine, and after entry into an insect host the IJs release the bacterial symbiont into the insect hemocoel. The insect dies within 48 h. The bacterium and nematode symbionts continue to grow and multiply within the infected insect until emergence of a new generation of IJs carrying the bacterial symbionts to initiate a new infection cycle in another insect.

A characteristic feature of this nematode–bacterium–insect interaction is that the infected insect cadavers do not putrefy [2], which is unlike the normal multiple microbial degradation of cadavers of insects that have died from other causes. This caused Dutky [2] to speculate that the symbiotic bacteria produce antimicrobial metabolites that prevent the growth of competing microorganisms and putrefaction of the nematode-infected insect cadavers. It is now known that antibiotic production is common to *Photorhabdus* spp. when cultured in vitro, and several antibiotics, such as stilbene derivatives, anthraquinone derivatives, genistin [3–5], a furan derivative and a phenol derivative, have been identified (Hu et al., unpublished).

Although this hypothesis was generally accepted when describing the natural situation in the tripartite, nematode–bacterium–insect interaction, it is based mainly on in vitro antibiotic bioassays [2,3,5–7], and there is little in vivo experimental evidence to support the hypothesis [8,9]. Jarosz [9] questioned this hypothesis and reported that only a low antibiotic potency of limited spectrum of antibacterial activity occurred throughout the entire development of the nematode in *Galleria mellonella* infected with *Steinernema carpocapsae* or *Heterorhabditis bacteriophora*. Consequently, Jarosz [9] proposed that the lack of putrefaction of the infected insect was due rather to rapid growth of the bacterial symbiont preventing or minimizing competition by secondary invaders of the insect cadaver. However, Hu et al. [10] reported that the antibiotic, 3,5-dihydroxy-4-isopropylstilbene (ST), was present at about 1500 and 4000...


μg g⁻¹ wet insect, respectively, in larval G. mellonella cadavers infected by the nematode, Heterorhabditis megidis 90, and 5 days post nematode infection. These concentrations of ST are many times higher than that needed to inhibit the growth of several species of soil bacteria and fungi under in vitro experimental conditions [5]. Hu et al. [11] studied the metabolic composition of the Photorhabdus–Heterorhabditis–Galleria interaction and found that the antimicrobials, ST, 3,5-dihydroxy-4-ethylstilbene and several anthraquinone derivatives, were major metabolic components of larval G. mellonella cadavers infected by H. megidis 90. In the same study the authors reported also small amounts of an unidentified antibiotic, AT, which since has been identified as a novel antibiotic, ace-oxide (Hu et al., unpublished). More recently, Hu et al. [12] reported that ST was commonly produced in larval cadavers of G. mellonella infected by each of five different Heterorhabditis–Photorhabdus associations. This series of in vivo studies provides evidence of the presence of relatively large quantities of antibiotics in the entomopathogenic nematode-infected cadavers and tends to support Dutky’s [2] hypothesis of antibiotic inhibition.

Despite the accumulating and sometimes conflicting information about this tripartite nematode–bacterium–insect interaction, the relationship between antibiotic production, the nematode and bacterial growth and the effect of the antibiotics on non-symbiotic bacteria within the infected cadaver is unclear. Consequently, experiments were done to investigate the time course of ST production during the growth and development of P. luminescens C9 and H. megidis 90 in larval G. mellonella.

2. Materials and methods

2.1. Nematode and insect

Heterorhabditis megidis 90, nematode symbiont of the bacterium, Photorhabdus luminescens C9, was originally collected from a soil sample in Summerland, BC, Canada. The nematode was maintained in the laboratory by passing through last-instar larval G. mellonella, which were supplied by the Insectary of the Department of Biological Sciences, Simon Fraser University. The IJs were collected on a Petri dish trap [13] within 4 days of their initial emergence from the nematode-infected G. mellonella cadavers and allowed to pass through two layers of wet-strength paper tissue (Kimwipes, Kimberly-Clark, USA) to ensure that only active IJs were collected. The collected IJs were surface sterilized with 0.2% thimerosal (Sigma), and suspended in phosphate-buffered saline (PBS) prior to injection into the insect hemocoel [14].

For each experiment, last instar larval G. mellonella (∼0.2 g larva⁻¹) were selected and the average weight (AW) of every five larvae was determined by weighing six groups of five randomly selected larvae. These larvae were used immediately after their selection.

2.2. Population dynamics of P. luminescens C9

Each selected G. mellonella larva was injected with 4 μl of PBS containing about 25 surface-sterilized IJs of H. megidis 90 and then incubated at 25°C in the dark. At 0, 6, 12, 24, 48 and 72 h after injection, and then on alternate days until day 27 after injection, five injected larvae were randomly collected, their body surface washed clean three times with tryptic soy broth (TSB, Difco), and then the larvae were homogenized with 2 ml of TSB in a small mortar. The macerated material was transferred to a measuring bottle and adjusted to 10 ml with TSB. Standard dilution–plating methods of the bacterial cells were followed immediately using the dyed TSB agar plates (TSB agar plus 25 μg bromothymol blue l⁻¹). After 48 h of incubation at 25°C in the dark, the number of colonies of P. luminescens per plate was recorded and converted to colony-forming units (CFU) g⁻¹ wet insect based on dilution times of the macerated material and the larval AW. The identity of the bacteria on the plates was confirmed using morphological, biochemical and physiological characteristics as defined by Thomas and Poinar [15,16] and Boemare and Akhurst [17]. The above process was performed under standard sterile conditions. The homogenization and dilution-plating were done immediately after sample collection at each sampling time. The experiment was repeated once, and five larvae injected with PBS alone served as a control in each experiment.

2.3. Production of ST by P. luminescens C9

The G. mellonella larvae were selected and AW determined as described above. Each of the larvae was then injected with surface-sterilized IJs of H. megidis 90 (∼25 IJs/larva), each of them was carrying the bacterial symbiont, P. luminescens C9, and kept in an incubator at 25°C in the dark. At each sampling time (0, 3, 6, 12, 24, 48 and 72 h, then every alternate day until 21 days, and also at 27 days after infection) three samples, each of five randomly selected larvae, were homogenized separately with acetone in a small mortar and, subsequently, a methanol extract was prepared as described by Hu et al. [10]. The concentration of ST in the methanol extract in each of the three samples at each sampling time was quantified using a TLC-UV method [10]. The amount of ST in each sample at each sampling time was determined by dividing the total amount of ST in each sample by the larval AW.

Fifteen larvae injected with only PBS served as the control. The experiment was repeated once except that the samples were collected at 1, 2, 3, 5, 7, 12, 17, 22 and 27 days after infection.
2.4. Development of H. megidis 90

To monitor the development of H. megidis 90 within nematode-infected G. mellonella larvae, three additional larvae were selected randomly every day from the above-injected larvae, dissected daily under the stereo microscope (25×) and the developmental stages of the nematodes were recorded. The experiment was repeated once.

2.5. Antibiotic activity of ST against gut bacterium from G. mellonella

The minimum inhibitory concentration (MIC) of ST against an unidentified gut bacterium from G. mellonella was determined by the liquid microdilution method in a 96-well plate. The final density of the bacterium was 1.0×10^5 cells ml^-1 in test solutions, which were prepared with TSB medium containing ST at concentrations of 100, 50, 25, 12.5, 6.25, 3.1, 1.5 or 0.75 μg ml^-1. The plate was incubated at 25°C in the dark without shaking, and the MIC was checked 24 h after incubation. The test was repeated once and streptomycin sulfate (Sigma) was included as a reference in each test.

3. Results

3.1. Population dynamics of P. luminescens C9

All larval G. mellonella infected with H. megidis 90 died by 48 h postinfection and turned reddish brown. Within 24 h of infection, the bacterial symbiont, P. luminescens C9, and an unidentified species of non-symbiotic bacterium were readily isolated from the infected insect larvae. The population of the non-symbiotic bacterium decreased during the first 24 h and was almost undetectable at 48 h. P. luminescens increased greatly to about 1.2–2.6×10^9 CFU g^-1 wet insect at 24 h postinfection (Fig. 1a). The population of the bacterial symbiont continued to grow and reached to 1.2–2.0×10^10 CFU g^-1 wet insect at about 7–9 days postinfection. Thereafter, it remained relatively constant until termination of the experiment at 27 days postinfection (Fig. 1a).

None of the control larvae died or changed color into reddish brown. They carried only the non-symbiotic bacterium in their gut, which formed small yellow colonies on TSB agar plates after incubation at 25°C. The results of the repeat experiment were similar (Fig. 1a).

3.2. Production of ST by P. luminescens C9

ST was not detectable in either experiment during the first 24 h of infection of larval G. mellonella by the H. megidis 90, but increased rapidly thereafter up to 5 days after infection (Fig. 1b). It then remained at a level of 3000–3600 μg g^-1 wet larvae until 21 days before declining gradually. The control larvae did not contain ST.

3.3. Development of H. megidis 90

By day 14, large numbers of new generation IJs had been produced. Population peaks of hermaphroditic females, amphimictic females and new IJs occurred at about 5, 11 and 14 days, respectively (Fig. 1a).

3.4. MIC of ST against the gut bacterium

The MIC was 12.5 μg ml^-1 for ST. Streptomycin sulfate
had a MIC of 25 μg ml⁻¹ under the same in vitro test conditions. The results were confirmed by a repeat test.

4. Discussion

The early and relatively large amount of ST produced and its continued presence over the entire period of nematode development may suggest that it plays a significant role in the tripartite interaction. During the first few hours post nematode penetration of the larval host, the non-symbiotic bacteria carried on the body surface of the nematodes are probably eliminated by the insect’s immune system. This contrasts with the bacterial symbionts which are somewhat resistant to the insect’s immune system or are not recognized as non-self [18,19]. Consequently, the symbionts multiply and rapidly build up high population levels (Fig. 1a) within 24 h of infection. Following death of the larva and subsequent rupture of the degenerating gut, bacteria are released from the insect gut into the hemocoe of the host where they compete for nutrients with the bacterial and nematode symbionts within the insect cadaver. However, the rise in concentration of the antibiotic ST at this stage, which had a MIC of 12.5 μg ml⁻¹ against the gut bacterium, is coincident with the disappearance of the larval gut bacterium after 48 h of infection. This effectively minimized this bacterial competition for resources for the symbionts.

The observations of Jarosz [9] regarding the low potency and a narrow spectrum of antibiotic activity in H. bacteriophora PLHb81–P. luminescens Plp73 infected G. mellonella larvae, contrasts with those reported here. This may be due to the fact that Jarosz tested only aqueous extracts of the infected cadavers. The results, therefore, represented the antibiotic activity of only hydrophilic metabolites. Many antibiotic metabolites produced by Photorhabdus spp. in infected G. mellonella larvae, such as stillbene derivatives, anthraquinone derivatives and AT, are lipophilic [11], and would not be detected in an antibiotic bioassay of the aqueous extracts of the cadaver. Hu et al. [12] showed that ST (665.2 μg g⁻¹ wet insect) was produced in larval G. mellonella infected by H. bacteriophora Oswego, the same nematode species as used by Jarosz [9]. These entomopathogenic nematode species consistently carry the same species of bacterial symbiont, P. luminescens [20,21]. Consequently, it is reasonable to expect that ST was produced in G. mellonella infected by H. bacteriophora PLHb81. This is supported by the description of Jarosz [9] that dead G. mellonella infected by P. luminescens Plp73, bacterial symbiont of H. bacteriophora PLHb81, were red in color. This is characteristic of insects infected by Heterorhabditis carrying Photorhabdus and is clear evidence of the presence of anthraquinone pigments and stilbenes since stillbene and anthraquinone derivatives consistently occur simultaneously under both in vitro and in vivo cultures of P. luminescens ([3,5,11]; Hu et al., unpublished).

It seems probable that the early and continued presence as well as the common production of antibiotics in the nematode–bacterium–insect interaction [11,12] has at least two functions. It helps to minimize competition from non-symbiotic bacteria, especially early in infection, and subsequently prevents microbial putrefaction of the nematode-infected insect cadaver, although antibiotic inhibition may not be the only mechanism involved. Inhibition of non-symbiotic bacteria is probably a competitive advantage for the highly specific and obligatory Heterorhabditis–Photorhabdus symbiotic association [19,22]. The differential immune response of an infected insect toward the symbiotic and non-symbiotic bacteria [18], and subsequent rapid build-up of a large population of symbiotic bacteria at an early stage of nematode infection as the insect dies, followed by the production of significant amounts of the antibiotics, creates a suitable environment for the growth of the bacterium–nematode complex within the insect cadavers.

The growth and development of the Heterorhabditis–Photorhabdus complex within an infected insect may not, however, be completely free of competition. Jackson et al. [23] reported that 10 out of 12 strains of Heterorhabditis were maintained in a dixenic association with Photorhabdus sp. and the bacterium Providencia (= Proteus) rettgeri after many years of in vivo subculturing in the laboratory. Babic et al. [24] found that 33% samples of H. indica, which were collected in the Caribbean islands, carried non-symbiotic bacteria, Ochrobactrum spp. It appears that a combination of the insect’s initial immune response and the symbiont derived antibiotics does not lead to a monoxenic bacterial condition within a nematode-infected insect in all situations. Microbial pathogens of insects are common, so it is irrational to expect that an insect’s immune system can prevent the invasion of all kinds of microorganisms encountered within an insect. G. mellonella has a relatively weak immune system [19]. Consequently, those bacteria that adhere to the nematode body surface and are brought into the insect during IJ entry, and those endogenous bacteria that released from the degenerating gut of the insect host have an opportunity for growth and some of them may be resistant to the antibiotics produced by the symbiotic bacteria. In this regard, the reported bacteria, P. rettgeri and Ochrobactrum spp. have been shown to be resistant to antibiotics produced by Photorhabdus [23,24].

Unlike the Heterorhabditis–Photorhabdus–insect interaction, there is little information about antibiotic production in relation to bacterial growth and nematode development in insects infected by Steinernema–Xenorhabdus, the other entomopathogenic nematode–bacterium complex. Study of the antibiotic production in Steinernema–Xenorhabdus–insect interaction would help to clarify the biological roles of antibiotics in these tripartite interactions. This
may lead to the development of more efficacious use of entomopathogenic nematodes as biocontrol agents of insect pests and may lead also to the discovery of novel antibiotics from the bacterial cultures.

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References