A new broad-spectrum protease inhibitor from the entomopathogenic bacterium *Photorhabdus luminescens*

Kevin E. Wee, Christopher R. Yonan and F. N. Chang

Author for correspondence: F. N. Chang. Tel: +1 215 204 8843. Fax: +1 215 204 6646.
e-mail: fchang@nimbus.temple.edu

Department of Biology,
Temple University,
Philadelphia, PA 19122,
USA

A new protease inhibitor was purified to apparent homogeneity from a culture medium of *Photorhabdus luminescens* by ammonium sulfate precipitation and preparative isoelectric focusing followed by affinity chromatography. *Ph. luminescens*, a bacterium symbiotically associated with the insect-parasitic nematode *Heterorhabditis bacteriophora*, exists in two morphologically distinguishable phases (primary and secondary). It appears that only the secondary-phase bacterium produces this protease inhibitor. The protease inhibitor has an M_r of approximately 12 000 as determined by SDS-PAGE. Its activity is stable over a pH range of 3–5–11 and at temperatures below 50 °C. The N-terminal 16 amino acids of the protease inhibitor were determined as STGIVTFKND(X)GEDIV and have a very high sequence homology with the N-terminal region of an endogenous inhibitor (IA-1) from the fruiting bodies of an edible mushroom, *Pleurotus ostreatus*. The purified protease inhibitor inactivated the homologous protease with an almost 1:1 stoichiometry. It also inhibited proteases from a related insect-nematode-symbiotic bacterium, *Xenorhabdus nematophila*. Interestingly, when present at a molar ratio of 5 to 1, this new protease inhibitor completely inactivated the activity of both trypsin and elastase. The activity of proteinase A and cathepsin G was partially inhibited by this bacterial protease inhibitor, but it had no effect on chymotrypsin, subtilisin, thermolysin and cathepsins B and D. The newly isolated protease inhibitor from the secondary-phase bacteria and its specific inhibition of its own protease provides an explanation as to why previous investigators failed to detect the presence of protease activity in the secondary-phase bacteria. The functional implications of the protease inhibitor are also discussed in relation to the physiology of nematode-symbiotic bacteria.

Keywords: protease, protease inhibitor, insect nematode, inhibitor–protease interaction

INTRODUCTION

Photorhabdus luminescens is an entomopathogenic bacterium symbiotically associated with insect-parasitic nematodes of the family Heterorhabditidae, which are used for biological control of many unwanted insects (Poinar *et al.*, 1977; Gaugler & Kaya, 1990). The bacteria are carried inside the gut of the non-feeding third-instar infective stage nematode (Poinar *et al.*, 1977; Poinar, 1990). Infection occurs when the insect-parasitic nematode invades the host insect through

natural openings such as the mouth, anus or cuticle followed by penetration into the haemocoel of the host. Once inside the haemocoel, the nematode secretes its bacterial symbiont. The bacteria then proliferate within the haemocoel, killing the host insect via septicaemia (Akhurst, 1980; Poinar, 1990). The bacteria also release secondary metabolites and hydrolytic enzymes such as protease, lipase and lecithinase which are essential for the development of progeny nematodes (Schmidt *et al.*, 1988; Boemare & Akhurst, 1988; Wang & Dowds, 1993).

Ph. luminescens bacteria occur in two phases, primary and secondary, which can be distinguished by bio-

Abbreviation: CBZ-Lys-pNP, *N*α-CBZ-L-lysine *p*-nitrophenyl ester.

chemical tests, bioluminescence and colony morphology (Akhurst, 1980; Boemare & Akhurst, 1988). Previous investigators have shown that the primary-phase bacteria are present predominantly in the gut of third-instar infective juvenile nematodes (Akhurst, 1980). It was also reported that only the primary-phase and not the secondary-phase bacteria possess the protease and this characteristic may be used to differentiate these two phases (Schmidt *et al.*, 1988; Nealson *et al.*, 1990; Forst & Nealson, 1996). Little is known about the regulation of protease activity in these bacteria. In this paper, we show that, contrary to previous reports, both primary- and secondary-phase bacteria synthesize protease. However, in the secondary-phase bacteria the protease activity is suppressed by this newly discovered protease inhibitor. We have purified and partially characterized this protease inhibitor with the aim of providing an explanation of its possible functions.

METHODS

Materials. *Photorhabdus luminescens* was obtained from surface-sterilized, infective stage *Heterorhabditis bacteriophora* HP88 nematodes. *Xenorhabdus nematophila* was kindly provided by Dr Thomas Schmidt (Indiana University, IN, USA). MacConkey agar was purchased from Sigma. Yeast extract and tryptone were obtained from Difco Laboratories. The Mini-Prep cell, Preparative IEF (Rotofor) unit and Electro-eluter were products of Bio-Rad Laboratories. Azocoll (50 mesh) was obtained from Calbiochem. Trypsin, elastase, proteinase A, cathepsin B, cathepsin D, cathepsin G, chymotrypsin, thermolysin, subtilisin, trypsin-acrylic beads as well as the substrates casein-FITC and *N* α -CBZ-L-lysine *p*-nitrophenyl ester (CBZ-Lys-pNP) were products of Sigma. Microcon 3 was purchased from Amicon. All other chemicals were of reagent grade and were obtained from local sources.

Purification of protease from primary-phase *Ph. luminescens*. The primary-phase bacteria were distinguished from the secondary-phase bacteria by colony morphology, presence of luminescence and the ability of the primary form to absorb neutral red from MacConkey agar (Boemare & Akhurst, 1988). Secondary-phase bacteria were isolated from *in vitro* cultures initiated from the primary-phase bacteria.

A single *Ph. luminescens* primary-phase colony was selected from a MacConkey agar plate and grown overnight in 5 ml Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). One millilitre of the inoculum was transferred into 1 l LB medium and incubated for 7 d at 25 °C in a shaker at 150 r.p.m. Unless otherwise stated, all subsequent operations were carried out in the cold (4 °C). Cells were removed by centrifugation at 7500 *g* for 60 min and the supernatant fraction was carefully collected into a large beaker. Ammonium sulfate was gradually added with stirring to the culture supernatant to 90% saturation. The precipitate was collected by centrifugation at 7000 *g* and then dissolved in 50 mM sodium phosphate buffer, pH 7.0. The ammonium sulfate precipitate was dialysed against distilled water overnight. The dialysate was then applied to a Bio-Rad Mini-Prep cell under native conditions and run at 100 V overnight with a flow rate of 0.75 ml min⁻¹. The fractions containing protease activity (as assayed in the next section) were collected and analysed for purity by PAGE in the presence of SDS. Protease from the *X. nematophila* primary-phase bacteria was also prepared according to the same procedure.

Assay of protease activity. Protease activity was determined initially using Azocoll as the substrate as described by Schmidt *et al.* (1988). Eppendorf tubes containing a reaction mixture (total volume 1.2 ml) consisting of 2 mg Azocoll ml⁻¹ in 50 mM sodium phosphate buffer (pH 7.0) were prepared. Enzyme solutions (10–200 μ l) were added to initiate the reaction and the mixture was then incubated at 37 °C. To terminate the reaction, the contents of each tube were centrifuged in an Eppendorf microcentrifuge for 2 min to remove any undigested Azocoll. The A_{520} of the liberated azo dye from the reaction supernatant was read using a Beckman DU-20 spectrophotometer. One unit of activity was defined as the amount of enzyme that caused an increase in the A_{520} of 0.01 min⁻¹ (Schmidt *et al.*, 1988).

Purification of protease inhibitor from secondary-phase *Ph. luminescens*. A tube containing 5 ml LB medium was inoculated with a single colony of secondary-phase *Ph. luminescens* isolated from a MacConkey agar plate and grown for 8 h at 28 °C with shaking at 150 r.p.m. One millilitre of this inoculum was transferred to a flask containing 500 ml LB medium and the culture was grown for 14 d at 28 °C with shaking at 150 r.p.m. The cells were removed from the culture medium by centrifugation at 7000 *g* for 1 h at 4 °C. Unless otherwise stated, all subsequent operations were carried out at 4 °C. The supernatant was collected and concentrated by adding ammonium sulfate with stirring to 90% saturation. The ammonium sulfate precipitate was collected by centrifugation at 9000 *g* for 1 h. The pellet was resuspended in 30 ml 50 mM sodium phosphate buffer (pH 7.0) and dialysed against 4 l distilled water overnight with two changes of water. The dialysate was then loaded into a Bio-Rad Rotofor unit, which separates proteins according to their isoelectric points in a liquid medium, using a pH gradient of pH 3–10. The collected fractions were analysed for inhibitor activity by mixing 50 μ l of each fraction with 50 μ l purified protease from the primary-phase *Ph. luminescens*. The fractions having the highest amount of inhibitor activity were incubated with trypsin-acrylic beads. The bound protease inhibitor was then eluted using the Electro-eluter. (In the presence of SDS, the Electro-eluter elutes the negatively charged proteins toward the anode, where they are trapped by a 3500 *M_r* cut-off dialysis membrane.) SDS was removed from the preparation by exchanging the lower buffer halfway through the run according to the manufacturer's protocol. The purity of the inhibitor was analysed using discontinuous SDS-PAGE. Protein concentration was determined by the Bradford microassay method (Bradford, 1976).

Stoichiometry of protease–protease inhibitor interaction. Ten microlitres of a 100 nM purified protease from primary-phase *Ph. luminescens* was incubated with various amounts of purified protease inhibitor from secondary-phase *Ph. luminescens* and allowed to react for 20 min at room temperature. Since the azocoll method used previously was rather insensitive, a different substrate (CBZ-Lys-pNP) was used for the stoichiometry study. Twenty microlitres of 50 μ M CBZ-Lys-pNP substrate was then added to the reaction mixture and further incubated for 10 min at 37 °C. The reaction was terminated by adding 700 μ l ice-cold 0.2 M sodium acetate buffer (pH 4.0) and the A_{326} was read immediately in a Beckman DU-20 spectrophotometer (Twining, 1994). Protease inhibitor activity was determined as the percentage of protease activity remaining when compared to the control tube containing buffer alone.

Activity spectrum of purified protease inhibitor. To study the activity spectrum of the purified protease inhibitor, it was

necessary to use a general protease substrate, and casein-FITC (Twining, 1994) was found to be an ideal substrate for this purpose. The following proteases were used: primary-phase proteases purified from both *Ph. luminescens* and *X. nematophila*, proteinase A, cathepsin B, cathepsin D, cathepsin G, chymotrypsin, elastase, thermolysin, subtilisin and trypsin. Each protease was diluted in 50 mM sodium phosphate buffer (pH 6.5) and adjusted to its pH optimum according to the protocol suggested by Sigma Chemicals, except for cathepsin D, which was diluted in 50 mM sodium acetate buffer at pH 3.5. The assay mixture contained 5 µl 20 nM protease and 5 µl 100 nM protease inhibitor. After incubating at room temperature for 20 min, 20 µl casein-FITC (1 mg ml⁻¹) and 100 µl 50 mM buffer at the appropriate pH were added. The mixture was then incubated at 37 °C for 30 min and the reaction was stopped by adding 100 µl 5% TCA. The undigested casein-FITC was removed by centrifugation at 14000 r.p.m. in an Eppendorf microfuge for 3 min. Two hundred microlitres of supernatant was transferred to a tube containing 500 µl 0.2 M sodium phosphate buffer (pH 7.8) to neutralize the TCA. Inhibitor activity was determined as percentage of activity remaining when compared to a control tube containing only buffer, using an Amicon fluorimeter (excitation at 492 nm and emission at 515 nm).

pH stability of the protease inhibitor. Five microlitres of protease inhibitor was diluted in 50 µl of buffers of overlapping pH ranges from pH 3.5 to 11.0 and incubated at room temperature for 1 h. Microcon 3 was then used to exchange buffers to 50 mM sodium phosphate buffer, pH 6.8. Residual inhibitor activity was assayed against purified protease from primary-phase *Ph. luminescens*. The assay contained 10 µl 10 nM enzyme and 50 µl 10 nM pH-adjusted protease inhibitor and the reaction mixture was incubated at room temperature for 20 min. Casein-FITC (20 µl) (1 mg ml⁻¹) and 40 µl 50 mM sodium phosphate buffer (pH 6.8) were then added and the reaction mixture was further incubated at 37 °C for 30 min. The reaction was then stopped with 100 µl 5% TCA and the undigested casein-FITC was removed by centrifugation at 14000 r.p.m. in an Eppendorf microfuge for 3 min. Two hundred microlitres of supernatant was transferred to a tube containing 500 µl 0.2 M sodium phosphate buffer (pH 7.8) to neutralize the TCA. Protease inhibitor activity was determined fluorimetrically relative to the control as described previously.

Thermal stability of protease inhibitor. Eppendorf tubes containing 5 µl protease inhibitor were incubated over a range of temperatures between 25 and 100 °C for 1 h and subsequently adjusted to 37 °C. The adjusted protease inhibitor was mixed with 10 µl primary-phase protease and the activity was assayed as described previously.

N-terminal peptide sequencing of purified inhibitor from *Ph. luminescens*. Protein sequence determination was performed by Dr Joe Lykam of Michigan State University Department of Macromolecular Biochemistry (East Lansing, MI, USA).

RESULTS

As indicated earlier, bacteria symbiotically associated with insect-parasitic nematodes exist in two morphologically distinguishable phases – primary and secondary (Boemare & Akhurst, 1988). Previous investigators have reported that only the primary-phase bacteria contained the protease activity (e.g. Schmidt *et al.*, 1988). While studying proteases from various nematode-symbiotic bacteria, we have confirmed that a number of

Table 1. Protease activity of nematode-symbiotic bacteria

One activity unit was defined as a change in A_{520} of 0.01 min⁻¹ (mg protein)⁻¹ using the azocoll assay as described in Methods. Each sample contained 33.3 µg total protein from cell-free culture supernatant. The experiment was performed in triplicate.

Bacterium	Phase	Protease activity (unit)
<i>X. nematophila</i>	Primary	0.959 ± 0.031
<i>X. nematophila</i>	Secondary	0.651 ± 0.005
<i>Ph. luminescens</i>	Primary	0.360 ± 0.013
<i>Ph. luminescens</i>	Secondary	0.017 ± 0.016

Fig. 1. SDS-PAGE zymogram of protease activity from both primary and secondary cultures of *X. nematophila* and *Ph. luminescens*. Ten microlitres of supernatant from 7-d-old cultures was used and loaded onto a 12% polyacrylamide gel in the presence of 0.05% gelatin and stained with Coomassie blue (Ong & Chang, 1997). Lanes: 1, protease from primary-phase *X. nematophila*; 2, protease from secondary-phase *X. nematophila*; 3, protease from primary-phase *Ph. luminescens*; 4, protease from secondary-phase *Ph. luminescens*.

secondary-phase bacteria had extremely low protease activity but were puzzled by high protease activity exhibited by other secondary-phase bacteria. For example, Table 1 shows that unlike *Ph. luminescens*, both primary and secondary phases of *X. nematophila* produced high protease activity. Analysis of both primary and secondary phases of *X. nematophila* and *Ph. luminescens* by zymography confirmed the presence of high protease activity in *X. nematophila* (Fig. 1). Unexpectedly, it also showed that the secondary phase of *Ph. luminescens* also contained very high protease activity (Fig. 1, lane 4). A possible explanation for this apparent contradiction is the presence of an inhibitor(s) in the secondary phase of *Ph. luminescens*. An attempt was therefore made to isolate such a protease inhibitor.

Table 2. Purification of protease inhibitor from secondary-phase *Ph. luminescens*

One unit of inhibitor activity was defined as the amount of protease inhibitor that caused a 50% reduction in protease activity when compared to a control at 520 nm using the azocoll assay as described in Methods. Specific activity was defined as units mg⁻¹.

Step	Fraction	Total volume (ml)	Total protein (mg)	Total units	Specific activity (units mg ⁻¹)	Purification (-fold)	Yield (%)
I	Crude extract	500.0	415.00	51875	125	1	100
II	(NH ₄) ₂ SO ₄ precipitation	48.0	144.00	37440	260	2	72
III	IEF	6.0	3.20	12308	3846	31	24
IV	Affinity chromatography	3.6	0.18	8280	46000	368	16

Fig. 2. SDS-PAGE analysis of protein composition at various stages of purification of the protease inhibitor. Lanes: 1, crude cell-free culture; 2, ammonium sulfate precipitate; 3, trypsin bead unbound fraction; 4, elution of bound proteins from trypsin beads. Visualization of proteins was carried out by silver staining.

Purification of the protease inhibitor from secondary-phase *Ph. luminescens*

Since protease inhibitor has not been reported in bacteria symbiotically associated with insect-parasitic nematodes, a purification scheme was developed using preparative isoelectric focusing and affinity chromatography. Both primary- and secondary-phase *Ph. luminescens* were examined for the presence of protease inhibitor activity. Protease inhibitor was only found in the secondary-phase bacteria. Table 2 shows that the protease inhibitor from the secondary-phase *Ph. luminescens* was purified 368-fold with a 16% yield. The purity of the protease inhibitor together with the protein composition at various steps of the purification process are shown in Fig. 2. The SDS-PAGE reveals that the purified inhibitor has an apparent M_r of approxi-

mately 12000. We have also confirmed that this protein band contained protease inhibitor activity by transferring it to nitrocellulose paper using capillary blotting followed by assaying for inhibitory activity using the procedure of Ohlsson *et al.* (1986) (results not shown).

pH and heat stability of the protease inhibitor

To test the pH stability of the protease inhibitor, it was incubated at room temperature for 1 h in the pH range 3.5–11.0. Residual inhibitor activity was measured as described in Methods. Interestingly, the protease inhibitor retained its full activity when it was exposed to the pH range tested. The thermal stability of the inhibitor was assayed within the range of 25–100 °C; it was inactivated when the temperature was above 50 °C (results not shown).

Determination of the N-terminal amino acid sequence of the protease inhibitor

After localization of inhibitory activity, the protease inhibitor band was excised from the polyacrylamide gel and subjected to amino acid sequence determination. The N-terminal 16 amino acids of the purified protease inhibitor were determined as STGIVTFKND(X)GEDIV (residue 11 appears to be modified). A sequence search was performed and it was found that it bears high sequence homology to the N-terminal region of an endogenous inhibitor (IA-1) from the fruiting bodies of an edible mushroom, *Pleurotus ostreatus* (Dohmae *et al.*, 1995). As shown below, 11 of the first 15 amino acids (73%) were identical between these two protease inhibitors.

<i>Pl. ostreatus</i>	S	A	G	K	F	I	V	I	F	K	N	D	V	S	E	D	K	I
<i>Ph. luminescens</i>	S	T	G	-	-	I	V	T	F	K	N	D	(X)	G	E	D	-	I

Interestingly, both protease inhibitors start with the serine residue, although in the mushroom it is acetylated, suggesting its cytosolic location (Odani *et al.*, 1999). The

Fig. 3. Inhibition of protease activity from *Ph. luminescens* by its homologous protease inhibitor. Primary-phase protease was incubated with various amounts of secondary-phase protease inhibitor and the remaining protease activity was assayed as described in Methods.

Table 3. Activity of protease inhibitor against various proteases

Various protease enzymes (homologous and heterologous) at a concentration of 20 nM were assayed against protease inhibitor from the secondary phase of *Ph. luminescens* (100 nM). Casein-FITC (1 mg ml⁻¹) was used as the substrate. The assay was read in an Amicon fluorimeter (excitation at 492 nm, emission at 515 nm) as described in Methods.

Protease	Inhibition (%)
<i>Ph. luminescens</i> (primary)	100 ± 3
<i>X. nematophila</i> (primary)	87 ± 5
Trypsin	100 ± 3
Elastase	100 ± 5
Proteinase A	68 ± 2
Cathepsin G	41 ± 5
Cathepsin B	0 ± 5
Cathepsin D	0 ± 3
Chymotrypsin	0 ± 3
Thermolysin	0 ± 3
Subtilisin	0 ± 3

Ph. luminescens protease inhibitor, on the other hand, is a secretory protein.

Protease–protease inhibitor interaction

In order to study the nature of the interaction between the newly isolated protease inhibitor and its substrates, it was necessary to purify proteases from different nematode-symbiotic bacteria. The culture medium from the primary-phase *Ph. luminescens* was subjected to ammonium sulfate precipitation, dialysis and preparative PAGE under native conditions as described in Methods. A single band containing protease activity was observed with an apparent M_r of 56000 (figure not shown). The ability of protease inhibitor from *Ph.*

luminescens to inhibit its homologous protease was then examined. Fig. 3 shows that at a molar ratio of one to one, 90% of the protease activity was inhibited, indicating an almost stoichiometric interaction between the protease and its inhibitor.

Protease from *X. nematophila* was similarly purified with an apparent M_r of 53000 (figure not shown). The effect of *Ph. luminescens* protease inhibitor on protease activity from *X. nematophila* and several other proteases was also carried out using a molar ratio of 5 to 1 in favour of protease inhibitor. Table 3 shows that the *X. nematophila* protease was 87% inhibited by the heterologous protease inhibitor from *Ph. luminescens*. Interestingly, the purified protease inhibitor was able to completely inhibit the activity of both trypsin and elastase. The activity of proteinase A and cathepsin G was partially inhibited. However, chymotrypsin, cathepsin B, cathepsin D, thermolysin and subtilisin were not affected by the protease inhibitor.

DISCUSSION

The demonstration that both primary- and secondary-phase bacteria produced the protease (Fig. 1) called into question the commonly held notion that only the primary-phase bacteria contain the protease activity (e.g. Schmidt *et al.*, 1988). The failure to detect protease activity in the secondary-phase bacteria by other investigators can now be traced to the presence of the newly discovered protease inhibitor that suppressed the protease activity. The function of proteases in mammalian systems has been well studied and they are reported to be involved in processes such as the blood clotting cascade, cell cycle, activation of precursor proteins, processing and degradation of proteins and cellular development (for a review, see Travis & Potempa, 2000). Proteases have also been implicated in morphological differentiation and mycelium growth of *Streptomyces albidoflavus*, a Gram-positive soil bacterium widely known for possessing a variety of hydrolytic enzymes (Kang & Lee, 1997). The expression of the protease gene in this bacterium is found to be dependent upon the proteinaceous nutrients in the environment (Taguchi *et al.*, 1998). Protease inhibitors have been suggested to modulate these protease-mediated reactions (Travis & Potempa, 2000).

Since *Ph. luminescens* is a symbiont of insect-parasitic nematodes, several properties of the protease inhibitor suggest that it may participate in the parasitization process. First, the protease inhibitor is rather unusual in that it retains full activity in the pH range tested (3.5–11.0). This broad pH range not only covers the pH of insect haemolymph, where the bacteria are initially released by insect nematodes, but also the gut pHs of various insects. It is known that both the lepidopteran and dipteran insects have rather alkaline guts whereas the coleopteran insects have acidic guts (McFarlane,

1985). The fact that *Ph. luminescens* protease inhibitor is active at these gut pHs indicates that it may interfere with the insect's digestive system and speed up the parasitization process. The exceedingly high homology between *Ph. luminescens* protease inhibitor and that produced by the fruiting bodies of an edible mushroom, *Pl. ostreatus*, used to defend against ingestion by insects, lends further support to the role of protease inhibitor in controlling the digestive processes of the insect host. Second, the protease inhibitor was found to suppress insect proteases that are required to activate the prophenoloxidase cascade essential for the insect defensive system (Kanost, 1999; Park *et al.*, 2000). Although we have not tested the activity of *Ph. luminescens* protease inhibitor against various insect proteases, the broad spectrum of the protease inhibitor (Table 3) suggests that it will inactivate the insect proteases.

The protease inhibitor may also be involved in regulating the bacterial phase shift during the development of insect nematodes. It is known that the primary-phase bacteria are present predominantly in the gut of insect-parasitic nematodes (Akhurst, 1980) and it is clearly advantageous to have high protease activity during the initial stage of the infection process inside a target insect host rich in proteinaceous nutrients. At later stages of infection when the nutrients are depleted, it will be necessary to control or curtail this protease activity. One way to regulate the protease activity is to convert some primary-phase bacteria, which apparently do not produce protease inhibitor or may have produced so small an amount that it escaped detection, into the secondary phase. It has been documented that when cultured *in vitro*, primary-phase *Ph. luminescens* can easily be converted to the secondary phase and this process appears to be irreversible (Bleakley & Nealson, 1988; Forst & Nealson, 1996). An alternative method for controlling the protease activity is to suppress the growth of the primary-phase bacteria at later stages of parasitization, thereby allowing the secondary-phase bacteria to populate. In this regard, Smigielski *et al.* (1994) showed that the secondary-phase bacteria can grow twice as fast *in vitro* when compared to the primary-phase bacteria. It has also been reported that in *Streptomyces albobogriseolus*, overproduction of protease (due to a deletion mutation in the protease inhibitor gene) leads to a marked decrease in growth rate (Taguchi *et al.*, 1998). It is likely that the secondary-phase *Ph. luminescens* bacteria without the burden of high protease activity can outgrow the primary phase and be dominant in a mixed culture when nutrients become limiting. Our results indicate that rather than being perceived primarily as an *in vitro* artefact, secondary-phase *Ph. luminescens* may actually be quite critical to the development of insect nematodes.

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