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Secreted proteases from *Photorhabdus luminescens*: separation of the extracellular proteases from the insecticidal Tc toxin complexes

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Abstract

Photorhabdus luminescens secretes both high molecular weight insecticidal toxin complexes and also a range of extracellular proteases into culture broth. Previous studies by others have suggested that insecticidal activity of the broth is associated with these proteases. However, by gene cloning and targeted knock-out, we have previously shown that oral insecticidal activity is associated with high molecular weight 'toxin complexes' (Tc) encoded by *toxin complex* or *tc* genes. Here we further clarify this distinction by biochemically separating the protease fractions away from the oral insecticidal activity of the Tc proteins. We purified three distinct protease fractions from the broth: one consisting of a single species of 55 kDa and two of several putatively related species of ~40 kDa. All of these clearly separate from the oral insecticidal activity associated with the high molecular weight Tc proteins and also show no effect on insect weight gain following injection into the haemocoel. Here we examine the substrate preferences and inhibitor profiles of these protease fractions and discuss their relationship with those previously described from other *P. luminescens* strains and phase variants. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Proteases; Insecticidal toxins; *Photorhabdus luminescens*; *Bacillus thuringiensis*

1. Introduction

Photorhabdus luminescens is a bacterium that lives in a symbiotic relationship with Heterorhabditid nematodes (Poinar et al., 1977). Following invasion of an insect host by the nematode, *P. luminescens* is released into the insect haemocoel where it is assumed to secrete a wide range of substances including lipases, proteases, antibiotics and lipopolysaccharides (Dunphy and Webster, 1988a,b; Schmidt et al., 1988; Yamanaka et al., 1992; Wang and Dowds, 1993; Clarke and Dowds, 1995), some of which are thought to be toxic to the insect (see Forst et al., 1997, for a recent review). *P. luminescens* can be readily cultured away from its nematode host and previous studies of culture supernatants have produced conflicting results on the role of the extracellular proteases in insect toxicity (Schmidt et al., 1988; Yamanaka et al., 1992; Bowen and Ensign, 1998; Jarosz,

1998). In previous work (Bowen et al., 1998) we have characterized the high molecular weight toxin complexes that are secreted into the growth media by *P. luminescens* strain W14 and which are orally toxic to our lepidopteran model *Manduca sexta*. The aim of the present paper was to purify the protease activity away from that of the Tc toxin complexes and therefore to facilitate a differentiation of both their oral and injectable toxicities.

The literature on the role of *Photorhabdus* and/or *Xenorhabdus* protease activity in insect toxicity is highly conflicting and often anecdotal. Schmidt et al. (1988) purified an alkaline metalloprotease from *P. luminescens* culture broth and inferred that proteases may have a role in insect toxicity via analogy with proteases produced by other insect pathogens. Jarosz et al. observed that both extracts of infected insects, and also cell free culture broths, were toxic to insects upon injection (Jarosz et al., 1991). Further, they suggested that this was correlated with the presence of the protease activity they observed in the cell free culture broth. In contrast, Yamanaka et al. made extracts from several *P. luminescens* strains, all of which contained high levels of protease activity but

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only one of which showed a low level of insect toxicity (Yamanaka et al., 1992). They therefore inferred that the protease activity was not correlated with insect toxicity. Further, Bowen and Ensign purified a high molecular weight protein fraction, which showed high oral toxicity to insects but failed to show any protease activity (Bowen and Ensign, 1998). Protease activities have previously been attributed to proteins of two different sizes, one estimated to be 57 kDa, which was secreted, and a second of 47 kDa, which was not secreted (Ong and Chang, 1997).

Our recent studies on *P. luminescens* have shown that oral toxicity to insects is associated with several high molecular weight toxin complexes (Tca, Tcb, Tcc and Tcd) secreted into the growth media (Bowen et al., 1998). In this study we were therefore interested in further characterizing the extracellular proteases of *P. luminescens* and in clarifying their role in oral and injectable toxicity to insects. Here we show that the protease activity of the cell free broth is associated with three protease fractions: one containing a single protein of 55 kDa and the other two containing a range of proteins of similar size of ~40 kDa. These three different fractions show differences in response to different substrates and inhibitors. However, importantly, the insecticidal activity of the high molecular weight toxin complexes is clearly separable from the protease fractions which are not toxic themselves either following ingestion or injection.

2. Materials and methods

Proteases were purified from culture broths of *P. luminescens* strain W14 (Bowen et al., 1998). Cultures were grown in a rotary shaker (225 rpm) for 120 h in 2% PP3 medium at 30°C. The cells were removed by centrifugation (10,000 g for 10 min). The cell free supernatant containing proteolytic activity was collected.

One liter of the cell free supernatant was loaded onto 80 ml of DEAE-Sephacel (Pharmacia) in a 2.6×20-cm bed. After washing the column with 50 mM K₂HPO₄, until the 280-nm absorbance returned to baseline, bound proteins were eluted with a linear gradient of 0–0.5 M KCl in 50 mM K₂HPO₄ over 180 min. Aliquots of 1.5 ml of proteolytically active fractions from this initial fractionation were subsequently loaded onto a 1.6×60-cm Sephacryl-200 column and eluted with 100 mM Tris-HCl (pH 8.0). Active fractions from gel-filtration were equilibrated with 15 mM triethanolamine-HCl (pH 7.5), and concentrated to ~5 ml in a 10-kDa centrifugal ultrafiltration device (Centriprep10, Amicon). These concentrated samples were then further fractionated by HPLC using a Vydac 300VHP575 strong anion exchange column; proteins were eluted with a linear gradient of 0–250 mM KCl in 15 mM triethanolamine-HCl (pH 7.5).

Oral bioassays following S-200 size-exclusion chromatography, were performed on neonate *M. sexta* larvae, as previously described (Blackburn et al., 1998; Bowen et al., 1998). Disks of 1 cm² of artificial diet were treated with 100 µl of individual fractions. Mortality was scored at 7 days. Assays of injectable activity in the different fractions were performed by injecting 5 µl of each of the HPLC fractions, each corresponding to 3 units/ml of relative activity against azocasein. Fractions were injected directly into the haemocoel of third instar *M. sexta* larvae using a 25-µl Hamilton syringe. Larval weight (mean and standard error) was assessed 72 h post injection after being held on artificial diet at 26°C.

Chromatographic fractions were assayed for proteolytic activity using azocasein (Sigma) as a substrate (Tomarelli et al., 1949). Aliquots of fractions (50 µl for DEAE and S-200 fractions, and 20 µl for HPLC fractions) were added to 5 mg of azocasein in 500 µl of Tris-buffered saline (pH 8) and incubated at 30°C for 4 h on an orbital shaker. Intact azocasein was precipitated by adding 500 µl of 10% trichloroacetic acid to the incubations and centrifuging at 10,000 g for 20 min. The absorbance values (440 nm) of the resulting supernatants were determined where increased absorbance indicates the presence of proteolytic activity.

HPLC purified proteases were also assayed using hide powder azure or HPA (Sigma) as a substrate. In these assays, a suspension of 5 mg of HPA in 500 µl of TBS was incubated with 20 µl of an HPLC fraction. After 4 h at 30°C, the incubations were centrifuged at 10,000 g for 20 min; the 595-nm absorbance of the resulting supernatants was then determined.

HPLC purified proteases were assayed against azocasein in the presence of the protease inhibitors: *o*-phenantroline, phosphoramidon, PMSF at 1 mM and EDTA at 1.5 mM. Assay conditions were otherwise identical to those described above. SDS-PAGE zymograms were performed as described by Schmidt et al. (1988) with minor modifications. Twelve percent polyacrylamide gels were co-polymerized with 0.05% gelatin or casein. Samples were mixed with SDS sample buffer without heat denaturation and run at 150 V. Following electrophoresis, the gels were washed for 4 h in 2.5% Triton X-100 in TBS with gentle agitation. Gels were incubated for an additional 4 h with several changes in TBS alone, then stained overnight in 0.1% amido black in 40% methanol and 10% acetic acid. Gels were destained in 10% acetic acid.

The pI of the 55-kDa protease was estimated from the predicted amino acid sequence of a DNA clone obtained from a W14 genomic plasmid library (our unpublished results). Presentation of this information in this paper on protease biochemistry was designed to facilitate comparisons with the pI estimates of protease fractions isolated by previous workers.

3. Results and discussion

After the release of *P. luminescens* into the insect haemocoel by the nematode, the bacteria appear to rapidly overcome the insect's defense mechanisms and kill the insect. During growth within the insect the bacterium is thought to release a variety of compounds including lipases, proteases, antibiotics and lipopolysaccharides (Forst et al., 1997). Previous studies on the extracellular proteases have been equivocal as to their role in insect toxicity. Thus, some authors have directly implicated them as toxic components, while others have suggested that they play a specific role in attacking antibacterial defense systems. Still others have found no correlation between extracellular proteases and insect toxicity (Schmidt et al., 1988; Yamanaka et al., 1992; Bowen and Ensign, 1998; Jarosz, 1998).

Following our recent work documenting the presence of four high molecular weight toxin complexes (Tca, Tcb, Tcc and Tcd) secreted by *P. luminescens* and the demonstration by genetic knockouts that Tca and Tcd are the major determinants of oral activity against Lepidoptera (Bowen et al., 1998), we were interested in clarifying the relationship between insect toxicity and the extracellular proteases. Here we show that the oral toxicity against *M. sexta* associated with the Tc complexes can be clearly separated from protease activity and that there appears to be no oral or injectable activity associated with the protease activities themselves. Thus, DEAE ion-exchange chromatography of culture supernatants revealed two well defined peaks of proteolytic activity, peaks A and B (Fig. 1a). When material from either of these peaks was further fractionated by size-exclusion chromatography (Fig. 1b), the proteolytic activity eluted as a single discrete peak. Peak A from the DEAE fractionation eluted from the S-200 column later than activity from peak B, suggesting an apparently lower molecular weight for A. Importantly, the bioassay of the resulting column fractions shows that, in both cases, the oral toxicity against *M. sexta* clearly separates from the peak of protease activity (Fig. 1c).

Biochemical separation of the protease activity finally resolved three fractions: one containing a single protein of 55 kDa (pI=4.59, estimated from the predicted amino acid sequence of a DNA clone, our unpublished results) and two others (termed 40A and 40B) containing multiple, putatively related proteins of approximately 40 kDa. Briefly, in the final chromatographic step, each of the resulting peaks from the S-200 column was further purified by HPLC using a strong anion-exchange column (Fig. 2a). For both peaks A and B, a major peak of absorbance (280 nm) coincided with a peak of proteolytic activity; as was the case for the initial DEAE fractionation. Peak A eluted later from the strong anion exchange column than peak B. HPLC fractionation of peak B indicated a second, later eluting region of weaker

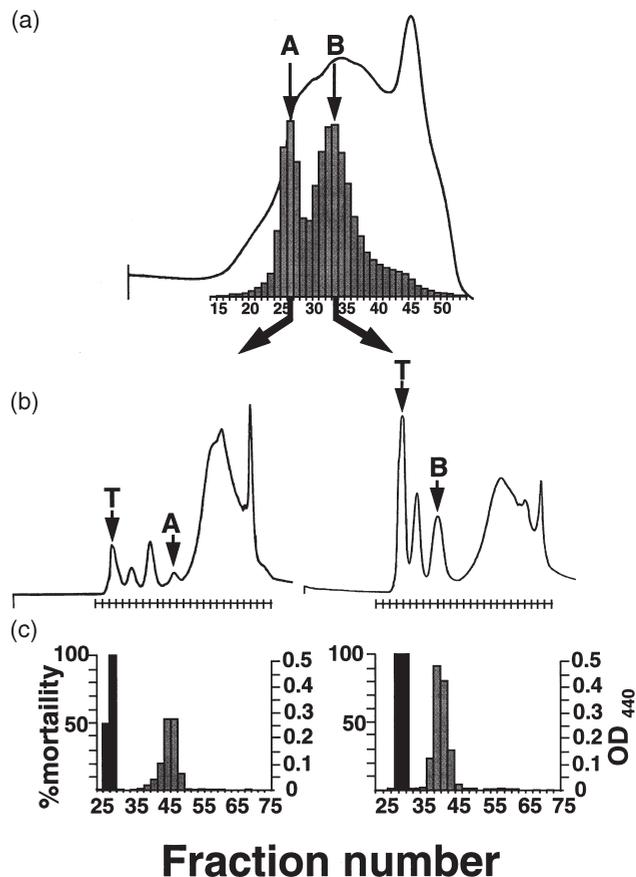


Fig. 1. Chromatography of *P. luminescens* broth separates protease activity from oral toxicity. (a) DEAE chromatography separates two peaks (A and B) of protease activity. (b) HPLC separation of each of the protease peaks. (c) Assay of HPLC fractions for both oral toxicity (% mortality to *M. sexta* neonates shown in black histogram) and protease activity (OD₄₄₀ shown in grey histogram). Note that the HPLC step resolves each protease peak into a separate peak of oral toxicity (T) and proteolytic activity (A or B).

proteolytic activity (Fig. 2b). The peak of proteolytic activity from HPLC fractionated peak A (Fig. 2a) resolves as a band of 55 kDa on both a gelatin (Fig. 3b) and casein (Fig. 3C) zymogram, whereas the two peaks of B resolve into two different protease fractions of approximately 40 kDa, termed 40A and 40B. Fraction 40A contains proteins of 35 and 38 kDa and 40B of 35 and 38 kDa, with an unresolved 'smear' of proteolytic activity from 38 to 44 kDa.

None of the protease fractions showed injectable activity (assessed as reduction in final larval weight) significantly different from those of the buffer injected controls (Fig. 4). This suggests that the protease fractions purified here have little or no direct toxicity to insects via injection. However, this simple injection assay does not exclude a role of these proteases in Tc toxin processing/activation and/or in overcoming insect immunity. The three protease fractions did show different activities against different protease substrates and also different inhibition profiles, with a range of inhibi-

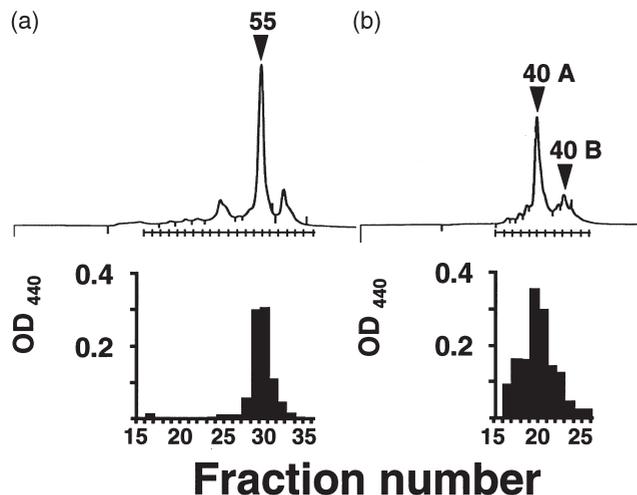


Fig. 2. Weak anion exchange HPLC separation of protease fractions. (a) Chromatogram from peak A and (b) chromatogram from peak B (for origin of peaks A and B see Fig. 1). Below each trace is shown the protease assay with azocasein. Note that peak A corresponds to a single peak of activity termed 55 kDa (arrow), whereas the protease activity of peak B is associated with a region corresponding to two HPLC derived peaks termed 40A (arrow) and 40B (arrow).

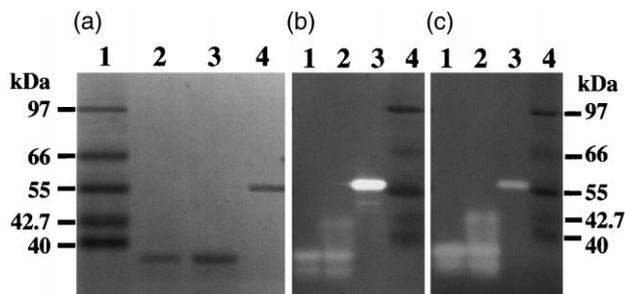


Fig. 3. Analysis of the different purified proteases by SDS PAGE and zymogram. (a) SDS PAGE coomassie stained gel of purified proteases. Lanes: (1) molecular weight standard, (2) 40A, (3) 40B and (4) 55-kDa protease fractions. (b) Gelatin zymogram showing region of clearing associated with protease activities. Lanes: (1) 40A, (2) 40B, (3) 55-kDa protease fractions and (4) molecular weight standard. (c) Casein zymogram (lanes as in panel b).

tors supporting the assumption that they contain a number of different enzymes. We quantitated the activity spectrum of an equal amount of these different proteases on the two different substrates, hide powder azure (HPA) and azocasein. The 40A protease fraction showed higher activity than the other proteases against HPA (Fig. 5a), whereas with azocasein the 55-kDa fraction and 40A fraction showed equal activity but at a higher level than the 40B fraction (Fig. 5b).

In order to further characterize the properties of the three protease fractions we also examined relative inhibition by four different protease inhibitors — phosphoramidon, phenanthroline, EDTA and PMSF (Fig. 5c). PMSF (a serine protease inhibitor) and phosphoramidon had little effect on any of the proteases. The metal chela-

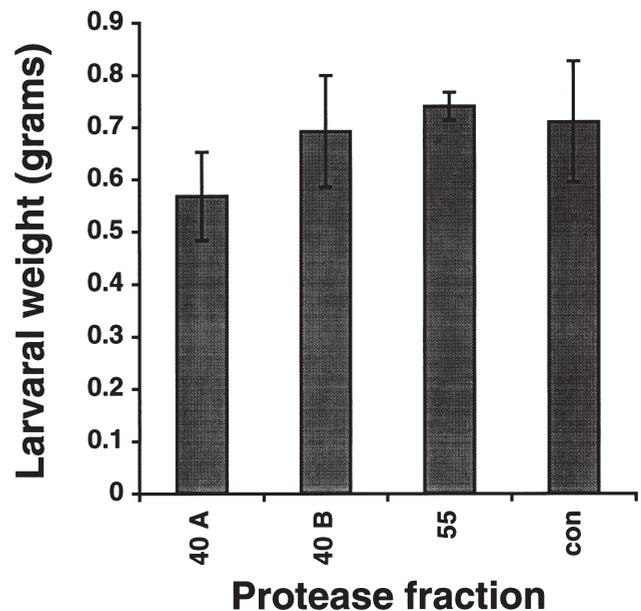


Fig. 4. Effect of the different protease fractions on the growth of *M. sexta* larvae 72 h post injection. Note that none of the different protease fractions reduces final weights significantly beyond those of the buffer only control (weights are shown with standard errors of the mean).

tors EDTA and phenanthroline inhibited the 55-kDa protease, suggesting that it is a metalloprotease. In contrast, although not strongly inhibited by EDTA, both the 40A and 40B protease fractions were inhibited by phenanthroline, suggesting that they may also be metalloproteases but with different inhibitor profiles than the 55-kDa protein.

These results are difficult to compare with those of other investigators due to differences in the strains used, the phase variants examined and also their culture conditions. Schmidt et al. described a 61-kDa protease from the *P. luminescens* strain Hm, grown in a gelatin broth (Schmidt et al., 1988). The protease was purified using a HPA assay to follow the proteolytic activity. The protease was found to have a pH optimum of 8, and was inhibited by *o*-phenanthroline and EDTA, but not by PMSF; on this basis, it was classified as an alkaline protease. Given the similarity of the inhibition profile and the closeness in size estimate, this is probably related to the 55-kDa protein described here.

Ong and Chang studied the effect of phase variation on secreted proteases from strains Hm and Hp using both conventional (one-dimensional) gelatin zymography and two-dimensional zymography (Ong and Chang, 1997). Proteases of 59 and 57 kDa were consistently observed from both primary and secondary phases of strains Hm and Hp. Two 'lower molecular weight protease bands' were also detected from primary phase cultures of strain Hp and were ascribed as degradation products of the 57- and 59-kDa proteins. The relationship between these two lower molecular weight proteases and the proteins from W14 is at present unclear.

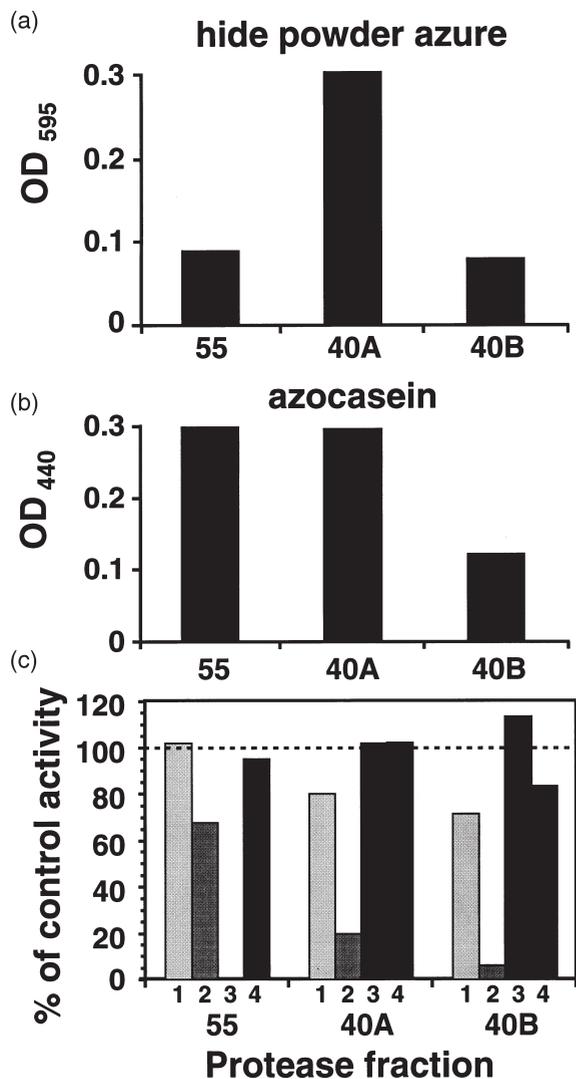


Fig. 5. Proteolytic activities and inhibitor profiles of the three different proteases. (a) Activity against hide powder azure (assayed at OD₅₉₅) and (b) against azocasein (assayed at OD₄₄₀). Note how the 40A protease fraction has high activity against HPA whilst both the 55 and 40B protease fractions have higher activity against azocasein. (c) Relative inhibition of the three different proteases by a range of protease inhibitors (scored as a percentage of uninhibited control activity). Bar number: (1) phosphoramidon, (2) phenanthroline, (3) EDTA and (4) PMSF.

The purification of the three different protease fractions described here has clearly demonstrated that they are *not* involved in the oral activity of *P. luminescens* broth which can be uniquely attributed to the Tc proteins. These fractions also appear to contain little, if any, injectable activity. In interpreting this analysis, we do stress that the protease substrates used here are general substrates (azocasein and hide powder azure) and therefore that we cannot eliminate the formal possibility that the Tc toxins themselves also have a specialized proteolytic activity. However, if they do, it certainly does not correspond to the bulk of the proteolytic activities measured by the previous investigators discussed above. The

mode of action of the Tc toxins themselves is currently under investigation in our laboratory.

In conclusion, the purification and separation of the general proteases secreted by *P. luminescens* described here will facilitate further examination of their potential roles in bacterial virulence: namely in potentially inhibiting antibacterial proteins such as cecropin (Jarosz, 1998) and/or in Tc toxin polypeptide cleavage (Bowen et al., 1998).

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References

- Blackburn, M., Golubeva, E., Bowen, D., ffrench-Constant, R.H., 1998. A novel insecticidal toxin from *Photorhabdus luminescens*: histopathological effects of toxin complex A (Tca) on the midgut of *Manduca sexta*. *Appl. Environ. Microbiol.* 64, 3036–3041.
- Bowen, D.J., Ensign, J.C., 1998. Purification and characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. *Appl. Environ. Microbiol.* 64, 3029–3035.
- Bowen, D., Rocheleau, T.A., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R., ffrench-Constant, R.H., 1998. Novel insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* 280, 2129–2132.
- Clarke, D.J., Dowds, B.C.A., 1995. Virulence mechanisms of *Photorhabdus* sp. strain K122 toward wax moth larvae. *J. Invert. Pathol.* 66, 149–155.
- Dunphy, G.B., Webster, J.M., 1988a. Lipopolysaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galleria mellonella* (Insecta: Lepidoptera) larvae. *J. Gen. Microbiol.* 134, 1017–1028.
- Dunphy, G.B., Webster, J.M., 1988b. Virulence mechanisms of *Heterorhabditis heliothidis* and its bacterial associate, *Xenorhabdus luminescens*, in the non-immune larvae of the greater wax moth, *Galleria mellonella*. *Int. J. Parasitol.* 18, 729–737.
- Forst, S., Dowds, B., Boemare, N., Stackebrandt, E., 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annu. Rev. Microbiol.* 51, 47–72.
- Jarosz, J., 1998. Active resistance of entomophagous rhabditid *Heterorhabditis bacteriophora* to insect immunity. *Parasitology* 117, 201–208.
- Jarosz, J., Balcerzak, M., Skrzypek, H., 1991. Involvement of larvicidal toxin in pathogenesis of insect parasitism with the Rhabditoid nematodes *Steinernema feltiae* and *Heterorhabditis bacteriophora*. *Entomophaga* 36, 361–368.
- Ong, K.L., Chang, F.N., 1997. Analysis of proteins from different phase variants of the entomopathogenic bacteria *Photorhabdus luminescens* by two-dimensional zymography. *Electrophoresis* 18, 834–839.
- Poinar, G.O., Thomas, G.M., Hess, R., 1977. Characteristics of the specific bacterium associated with *Heterorhabditis bacteriophora* (Heterorhabditidae: Rhabditida). *Nematology* 23, 97–102.
- Schmidt, T.M., Bleakley, B., Neilson, K.H., 1988. Characterization

- of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. *Appl. Environ. Microbiol.* 54, 2793–2797.
- Tomarelli, R.M., Charney, J., Harding, M.L., 1949. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. *J. Lab. Clin. Med.* 34, 428–433.
- Wang, H., Dowds, B.C.A., 1993. Phase variation in *Xenorhabdus luminescens*: cloning and sequencing of the lipase gene and analysis of its expression in primary and secondary phases of the bacterium. *J. Bacteriol.* 175, 1665–1673.
- Yamanaka, S., Hagiwara, A., Nishimura, Y., Tanabe, H., Ishibashi, N., 1992. Biochemical and physiological characteristics of *Xenorhabdus* species, symbiotically associated with entomopathogenic nematodes including *Steinernema kushidai* and their pathogenicity against *Spodoptera litura* (Lepidoptera: Noctuidae). *Arch. Microbiol.* 158, 387–393.