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Expression of *prtA* from *Photorhabdus luminescens* in *Bacillus thuringiensis* enhances mortality in lepidopteran larvae by sub-cutaneous but not oral infection

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ABSTRACT

The *prtA* gene from *Photorhabdus luminescens* encodes the virulence factor Protease A. When *P. luminescens* is injected into the hemocoel of insects by entomopathogenic nematodes, PrtA is a key component of pathogenicity thought to help degrade the immune system. The *prtA* gene was cloned and introduced on a plasmid into *Bacillus thuringiensis*. PrtA was shown to be actively expressed *in vitro* by cleavage of a specific Dabcyl–Edans heptapeptide substrate. There was no difference in the speed or level of mortality when spores and δ-endotoxins crystals of the transformed strain were fed to larvae of *Pieris brassicae*, as compared to the wild-type strain. When vegetative cells were injected into the hemocoel of larvae of *Galleria mellonella*, however, there was a significant increase in the rate and level of mortality over the wild type. The yield of *B. thuringiensis* per cadaver was a hundred-fold greater in the PrtA-secreting strain. The increased pathogenicity from intrahemocoelic infection may have been due to a greater ability to overcome the immune response of *G. mellonella* while other factors such as resident gut bacteria may have negated this advantage after oral dosage.

1. Introduction

Photorhabdus luminescens and *Xenorhabdus nematophila* are symbionts of entomopathogenic nematodes: the nematodes inject the bacteria into the hemocoel of target invertebrates where the bacteria bring about immunosuppression, septicemia and the subsequent death of the host (Eleftherianos et al., 2010). A number of pathogenicity determinants have been identified in these two Gram-negative species including hemolysis factor (Ribiero et al., 2003), hydrolases, lipopolysaccharide, regulatory factors, toxins and proteases (ffrench-Constant et al., 2007; Nielsen-LeRoux et al., 2012). Four proteases have been identified in *Photorhabdus* spp., the first one secreted during infection being PrtA, a zinc-requiring peptidase of the serralysin group (Marokházi et al., 2004). PrtA has been shown to have a very specific amino acid cleavage site which makes it amenable to assay with a fluorescent peptide substrate (Marokházi et al., 2007). Its mode of action may be immunosuppression, as evidenced by its natural substrates (Felföldi et al., 2009) and the fact that its production coincides with the early stages of infection rather than the degradation of the cadaver (Massaoud et al., 2010; Mustafa et al., 2010). Unlike *P. luminescens*, the invertebrate pathogenicity of *Bacillus thuringiensis* originates not from its direct inoculation into the hemocoel but from the ingestion of δ-endotoxins (van Frankenhuysen, 2009). Subsequently, there is a greater or lesser involvement of other virulence factors produced by the vegetative cells once the spores, ingested with the δ-endotoxins, have germinated (Dubois et al., 2012).

There is a growing understanding of the numerous obstacles to the bacterial pathogens of invertebrates successfully killing their hosts (Nielsen-LeRoux et al., 2012). Once *P. luminescens* and *B. thuringiensis* have reached the hemocoel, however, they face the same immune defenses and also the need to obtain nutrients and degrade host tissue.

One method of increasing pathogenicity and further understanding the mechanisms involved is to augment the virulence determinants of one invertebrate pathogen with a pathogenicity factor from another pathogen. An example of this is the oral co-administration to caterpillars of δ-endotoxins from *B. thuringiensis* with cells of *X. nematophila* (BenFarhat et al., 2013). Here the converse situation is reported with the heterologous expression of the *prtA* gene in *B. thuringiensis* and its effect on oral and sub-cutaneous dosage to lepidopteran larvae.

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2. Methods and materials

2.1. Bacterial strains and vectors

B. thuringiensis strain 158-S-2 is an environmental strain isolated from clover leaves (Bizzarri et al., 2008). *B. thuringiensis* strain HD-1 was obtained from Dr. Denis Burges (HRI, U.K.). *P. luminescens* was a gift from Dr. David Clarke (University of Cork, Ireland). Bacteria were cultured in Tryptone Soya Broth (Oxoid) unless stated otherwise.

2.2. Cloning of prtA gene

A cosmid library of the genome of *P. luminescens* in *Escherichia coli* was a gift from Dr. Nick Waterfield, University of Warwick, U.K. DNA from this library was extracted by heating colonies in sterile water at 99 °C in screw-topped Eppendorf tubes for 10 min. These were then centrifuged at 13,000*g* for 5 min and the supernatant (5 μL) used as the template in PCR reactions using the *prtA* primers (forward: 5′-ACTAGTATGTCTTTAAAGAAGAAA ATTTCA and reverse 5′-TCTAGATTATGAAAGAATGAAATCAGACT), designed against the full *prtA* gene of *P. luminescens* (EMBL accession number: AY928067.1). The thermal cycling profile was: 94 °C, 2 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 7 min. An amplicon of the correct size (approximately 1600 bp) was produced from one cosmid clone. This was cloned into the TOPO-TA plasmid (Invitrogen) in competent *E. coli* cells (Invitrogen), according to the supplier's instructions, and sequenced (Beckman, Coulter Cogenics, U.K.) to verify its identity. The *vip* promoter (Mesrati et al., 2010) from *B. thuringiensis* HD-1 was amplified using the temperature profile above and the forward (5′-TGCGGGCCTTAATTAATTCCGAAAAGT AGAATAAGCAAAATT) and reverse primers (5′-CTAGCGGCCGCGCT AGCTTGGTAAGGCTCTTGTGCTTAAT). These added an *Apa*I site to the 5′ end of the amplicon and a *Not*I site to the 3′ end. Complementary oligonucleotides for the *NheA* signal sequence were synthesized by Sigma Genosys (U.K.) with the addition of 5′ *Not*I and 3′ *Eco*RV sites (5′-GGAGCGGCCGCATGAAAAAAACTTTAATTACTG GATTATTAGTAACTGCTGTTAGTACTAGTTGTCCAATTCCAGTTAGTGC TTATGCTAAGATATCGG) (Fagerlund et al., 2010). Complementary oligonucleotides of the *rrn* terminator (Reeves et al., 1999) with 5′ *Sal*I and 3′ *Pst*I sites (5′-AAAACAAAGGCTCAGTCGGAA GACTGGGCCTTTTGTTT) were similarly purchased. Each pair of complementary oligonucleotides was annealed. The resulting double stranded *NheA* signal sequence was digested with *Not*I and ligated to the similarly digested *vip* amplification product. The resulting construct of the promoter and signal sequence regions was purified with a PCR clean-up kit (Macherey–Nagel) and digested with *Apa*I and *Eco*RV. This was ligated upstream of the *prtA* gene in the TOPO-TA plasmid. The promoter-signal sequence-*prtA* gene construct was PCR amplified using the flanking M13 sites in TOPO-TA. The amplicon was blunt-ended and ligated into the shuttle vector pHY300PLK (Takara, Japan) that had been digested with *Sma*I. The double-stranded *rrn* terminator was cloned between the *Sal*I and *Pst*I sites of pHY300PLK, downstream of the *prtA* gene. The entire insert was sequenced to ensure that it was correct and, in particular, that the signal sequence was in the correct reading frame with the ATG start site of the *prtA* gene.

2.3. Electroporation and expression of PrtA

The method of Bone and Ellar (1989) was used for *B. thuringiensis* transformation except that electroporation was carried out in 4 mm gap cuvettes (Microbiosystems) while using the same voltage. In addition, the bacteria were incubated after electroporation in Brain Heart Infusion (BHI) broth (Oxoid, U.K.) containing 0.5% glycerol (Sigma, U.K.) for 90 min at 30 °C, with shaking, before plating onto BHI agar containing tetracycline (5 μg mL^{-1}). Spore and δ-endotoxin crystal yields were determined as described by Johnson et al. (1998).

To check the stability of the *prtA* gene, 46 colonies of *B. thuringiensis* which had been recovered from cadavers of *Pieris brassicae* following oral infection (Section 2.5), were grown to sporulation. Following germination of these isolates they were subjected to PCR using the *prtA* primers (Section 2.2).

2.4. In vitro assay of PrtA

A fluorescence-quenched, peptide substrate, Dabcyl-glutamate-valine-tyrosine-alanine-valine glutamate-serine-Edans, (Dabcyl–EVYAVES–Edans) was synthesised by Activotec (Cambridge, U.K.). This is a specific substrate for Prt A (Marokházi et al., 2007). The assay method described by these authors was used except that cells of *B. thuringiensis* and *P. luminescens* in exponential phase were inoculated at 10% (v/v) into fresh medium and incubated at 30 °C, with shaking, at 150 r.p.m. Samples were taken at subsequent time points (Fig. 1), centrifuged for 3 min at 13,000*g*, resuspended in assay buffer (Marokházi et al., 2007) and washed in assay buffer. The cells were resuspended in assay buffer containing a cocktail of protease inhibitors (Roche) with Zn^{2+} ions added to give a free concentration of 1.5 mM. The cell suspensions from different growth stages were equalised spectrophotometrically to produce a density of 1×10^7 CFU mL^{-1}. The Dabcyl–EVYAVES–Edans substrate was added at a concentration of 2 mM and the assays were incubated in the dark for 60 min at 30 °C, with shaking. The cells were removed by centrifugation at 13,000*g* for 3 min and the supernatant assayed in a spectrofluorimeter (Zeiss) with an excitation wavelength of 340 nm and an emission wavelength of 495 nm. The control was *B. thuringiensis* carrying the pHY300PLK plasmid with no insert. Both isolates of *B. thuringiensis* were grown in TSA with tetracycline (5 μg mL^{-1}) at 28 °C, with shaking.

2.5. Bioassays

Third instar larvae of *P. brassicae* were obtained from Dr. Amit Prabhakar, University of Greenwich. Groups of 30 larvae were fed cabbage leaves coated with different amounts of spore and crystal

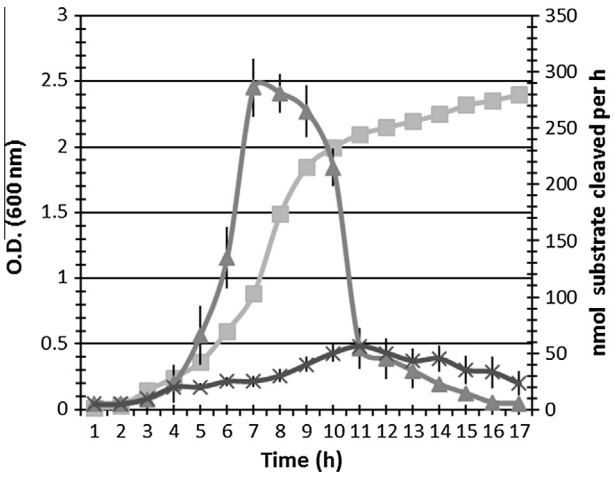

Fig. 1. PrtA activity against the Dabcyl–EVYAVES–Edans synthetic substrate. Representative growth curve (O.D. 600 nm), ■; PrtA activity in *B. thuringiensis* strain expressing *prtA* gene in plasmid pHY300PLK ▲, and PrtA activity in *B. thuringiensis* carrying plasmid pHY300PLK with no insert, X. The error bars represent 95% confidence intervals of the means of data from three experiments.

suspensions by the method of Bizzarri and Bishop (2007). Larvae of the waxworm, *Galleria mellonella* (Porton Aquatics, U.K.) were fed on a grain and honey diet (Fröbius et al., 2000). Thirty, final instar individuals with an average weight of 0.30 g (±10%), were injected in the base of the first proleg with 10 μL of a suspension of vegetative bacteria containing 5×10^4 CFU of either *B. thuringiensis* expressing *prtA* or containing the unmodified plasmid. The same number of control larvae were injected with sterile, phosphate-buffered saline (PBS). The insects were maintained at 20 °C and 70% humidity. Each bioassay was carried out three times with fresh materials. Statistical analysis was carried out with the SPSS 12.0.0 package.

Ten cadavers from each experiment were homogenised in a pestle and mortar and serially diluted in PBS at the end of the bioassays. The dilutions were then plated onto TSA with and without tetracycline (5 μg mL^{-1}). The results were expressed as CFU per cadaver.

3. Results

3.1. Cloning and expression of prtA in B. thuringiensis

Sequence analysis of the positive TOPO-TA clones showed an open reading frame with complete homology to the full (1455 bp) *prtA* gene of *P. luminescens* (accession number AY928067.1). There was no effect on bacterial physiology such as sporulation level, growth rate or δ-endotoxin yield between *B. thuringiensis* strain 158-S-2 secreting the PrtA protein as opposed to that carrying the plasmid without insert. Although pHY300PLK is a high copy number plasmid (Fukuda et al., 2009) the presence of tetracycline as the selection antibiotic resulted in curved and twisted vegetative cells, regardless of whether the plasmid contained the *prtA* gene or not. This was evident even at lower antibiotic concentrations.

The onset of vegetative insecticidal protein (VIP) production, which is controlled by the *vip* promoter used in this study, typically occurs from the middle of the vegetative phase (Estruch et al., 1996; Mesrati et al., 2010). PrtA activity appeared from early exponential phase and diminished at stationary phase (Fig. 1). This earlier appearance could be a result of the copy number of the pHY300PLK plasmid. The highest level of PrtA activity from *P. luminescens* under the same conditions was 1.1 μmol peptide cleaved h^{-1}. This was 56% of the maximum level measured from *B. thuringiensis* expressing *prtA*. The addition of the cocktail of protease inhibitors increased the activity of PrtA, as measured *in vitro* (data not shown). This was possibly as a result of the other proteases that *B. thuringiensis* produces being prevented from degrading the PrtA protein.

3.2. Bioassays

There was no significant difference in the rate or level of mortality compared to the strain carrying pHY300PLK alone when spores and crystals of *B. thuringiensis* expressing *prtA* were fed to *P. brassicae*. Similarly, the total count of *B. thuringiensis* per cadaver was the same from both preparations at 1.5×10^7 (s.d. = 7.2×10^6). When vegetative cells expressing PrtA were injected into *G. mellonella*, however, a more rapid onset and higher level of mortality ($p < 0.001$) was observed (Fig. 2). There was a significant difference ($p < 0.001$, Student's *t* test) between the yield per cadaver of *B. thuringiensis* from the strain expressing prtA, 1.23×10^{10} CFU (s.d. = 2.34×10^9), compared to that carrying the plasmid alone, 1.37×10^8 CFU (s.d. = 1.52×10^7). No other tetracycline-resistant bacterial species were recovered from the cadavers, even at lower dilutions. The counts of tetracycline-sensitive bacteria per cadaver

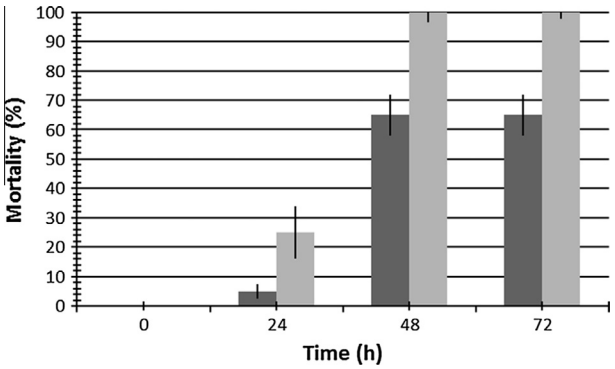

Fig. 2. Level of mortality of wild type- and PrtA-expressing *B. thuringiensis* vegetative cells injected into larvae of *G. mellonella*. Wild type- ■ and □ PrtA-expressing vegetative cells. The error bars represent 95% confidence intervals of the means of data from three experiments.

were 1.08×10^8 CFU (s.d. = 2.2×10^7) for *B. thuringiensis* expressing *prtA* and 9.51×10^8 CFU (s.d. = 5×10^8) in the control which just contained the unmodified plasmid. In comparison, larvae that had been injected with sterile PBS had a total bacterial count per cadaver of 1.6×10^7 CFU (s.d. = 4.1×10^6).

All 46 of the *B. thuringiensis* colonies randomly selected from those recovered from the *P. brassicae* cadavers produced the correct-sized amplification product with the *PrtA* primers. Further evidence that the plasmid was stably maintained during the course of the bioassays is that the number of *B. thuringiensis* colonies that appeared from the homogenates of both species of insect was very similar on both unsupplemented TSA and that containing tetracycline.

4. Discussion

Appreciable levels of PrtA activity were produced by heterologous expression in *B. thuringiensis* (Fig. 1). Activity *in vitro* peaked at almost twice the maximum level achieved by *P. luminescens* under similar conditions. Further comparisons are difficult because of differences in the genetic environment: the *prtA* gene in *B. thuringiensis* was carried on a high copy number plasmid (Fukuda et al., 2009) while, on the other hand, no attempt was made to optimise the codon usage: the GC ratio of the *prtA* gene is 44% which is noticeably higher than the GC ratio of the genome of *B. thuringiensis* (about 35%). Other factors were also involved. The secretion level of PrtA was greatly decreased when the VIP signal sequence (Shi et al., 2004) was used in place of the NheA signal sequence (data not shown). The reason for this is not known and it was not expected, particularly because the *vip* promoter appeared to function effectively. *B. thuringiensis* is notable as a producer of high levels of proteases (Ivanova et al., 2003). A cocktail of protease inhibitors was added to the PrtA assay mix to decrease the degradation of this enzyme by endogenous proteases. Zinc ions were added to overcome the presence of EDTA and so provide the optimal concentration for PrtA activity (Marokházi et al., 2007).

The lack of difference in the mortality and yield of *B. thuringiensis* in cadavers of *P. brassicae* after oral dosage with a strain secreting PrtA and the control may be due to the effect of resident gut bacteria. There is debate about the role of gut bacteria in pathogenesis of different lepidopteran species following infection with *B. thuringiensis* (for example, Johnston and Crickmore, 2009; Takatsuka and Kunimi, 2000). It is conceivable that, in the presence of a mixed infection, any additive pathogenic effect of PrtA secretion was inconsequential. There was, however, a significant difference in the rate and extent of mortality and also the yield of *B.*

thuringiensis cells when sub-cutaneous injection of *G. mellonella* was examined (Fig. 2). This is assumed to be due to the effect of PrtA debilitating the innate immunity of the larvae and/or increased degradation of the cadaver. It is surprising, however, that there was a hundred-fold greater yield of cells expressing PrtA in the cadavers compared to the control. Sporulation was complete in both cases so the lower yield in the control strain could not be due to delayed replication. Some residual, innate immune effectors might have decreased replication in the cells not expressing *prtA* although *B. thuringiensis* is highly developed to reproduce in cadavers (Dubois et al., 2012).

B. thuringiensis produces a number of pathogenicity factors (Guttmann and Ellar, 2000; Nielsen-LeRoux et al., 2012) beyond its pore-forming toxins. These include a number of proteases. One in particular, InhA (Fedhila et al., 2002) degrades antimicrobial peptides (Dalhammar and Steiner, 1984; Dubois et al., 2012) and allows spores to escape from phagocytes (Ramarao and Lereclus, 2005). Given the similar role predicted for PrtA it is not obvious why its production increased the rate and level of mortality in *G. mellonella* larvae. Similarly, however, *B. thuringiensis* secretes a number of chitinases (Nielsen-LeRoux et al., 2012) but when it was supplemented with chitinase from *Serratia marcesens* there was a significant increase in toxicity to larvae of *G. mellonella* and *Drosophila melanogaster* (Ozgen et al., 2013).

The synergistic toxicity to lepidoteran larvae of *B. thuringiensis* δ-endotoxins and cells of *Xenorhabdus* or *Photorhabdus* spp. has already been demonstrated (Jung and Kim, 2006; BenFarhat et al., 2013). Here a converse synergism is shown where *B. thuringiensis* was the infective agent and the additional virulence factor came from *P. luminescens*. This may have relevance in the co-application of the two pathogenic bacteria as biopesticides for recalcitrant pests (BenFarhat et al., 2013). Isolates with increased protease activity might, as a result of the higher yield per larva, be able to maintain a greater presence in the environment.

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