Genetic and biochemical characterization of PrtA, an RTX-like metalloprotease from *Photorhabdus*

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Repeats-in-toxin (RTX) toxins are a large family of calcium-dependent, pore-forming cytotoxins produced by different genera of the Enterobacteriaceae and Pasteurellaceae (Welch, 1991). RTX toxins are metzincin metalloendopeptidases secreted to the external medium via a Type I pathway. These proteases have been identified in *Erwinia chrysanthemi*, *Pseudomonas aeruginosa* and *Serratia marcescens*. The prtA gene carries the characteristic RTX repeated motifs and predicts high similarity to proteases from *Erwinia chrysanthemi*, *Pseudomonas aeruginosa* and *Serratia marcescens*. The prtA gene resides in a locus encoding both the protease ABC transporter (prtBCD) and an intervening ORF encoding a protease inhibitor (inh). PrtA activity is detectable 24 h after artificial bacterial infection of an insect, suggesting that the protease may play a key role in degrading insect tissues rather than in overcoming the insect immune system. Purified PrtA also shows cytotoxicity to mammalian cell cultures, supporting its proposed role in bioconversion of the insect cadaver into food for bacterial and nematode development.

The aim of the current study was to clone and characterize the zinc alkaline metalloprotease secreted by two species of *Photorhabdus*, *Photorhabdus luminescens* and *Photorhabdus*

INTRODUCTION

Zinc metalloproteases are common in pathogenic bacteria and are often attributed roles in virulence (Miyoshi & Shinoda, 2000). These include: (i) maturation of other enzymes, for example *Listeria monocytogenes* phospholipase C requires a zinc metalloprotease for activation (Miyoshi & Shinoda, 2000; Ravenneau et al., 1992); (ii) direct toxin activity, as is the case for the enterotoxin of *Bacteroides fragilis* (Kling et al., 1997; Moncrief et al., 1995), the *Bacillus anthracis* lethal toxin (Hammond & Hanna, 1998; Hanna, 1999) and *Clostridium* spp. neurotoxin activity (Tonello et al., 1996); and (iii) degradation of host connective tissues, which has also been associated with zinc metalloprotease activity, for example in *Pseudomonas aeruginosa* (Olson & Ohman, 1992) and *Clostridium histolyticum* (Yoshihara et al., 1994).

Abbreviations: MFP, membrane-fusion protein; OMP, outer-membrane protein; RTX, repeats-in-toxin.

The GenBank accession numbers for the W14 and K122 prtA clone sequences reported in this paper are AY230749 and AY230750 respectively.
temperata (Fischer-Le Saux et al., 1999), represented by the strains, P. luminescens subsp. akhurstii strain W14 (Bowen et al., 1998) and Photorhabdus temperata strain K122 (Waterfield et al., 2001), hereafter referred to simply as strains W14 and K122 for clarity. Photorhabdus is an entomopathogenic member of the Enterobactericeae found in a symbiotic relationship with entomopathogenic nematodes of the family Heterorhabditiae (Forst & Clarke, 2001; Forst et al., 1997). The bacteria occupy the intestinal tract of the infective stage of the nematode, the infective juvenile. The nematodes live in the soil where they actively seek out potential insect larval hosts. On finding a host, the nematode enters the larva and regurgitates the bacteria into the insect haemocoel (open circulatory system). The bacteria then grow rapidly producing a wide range of toxins and hydrolytic exoenzymes that kill the insect and aid in the conversion of the cadaver into food (‘bioconversion’) for both the bacteria and developing nematodes (ffrench-Constant et al., 2003).

Previous attempts to characterize proteolytic activities secreted by either Photorhabdus or Xenorhabdus have led to considerable confusion in the literature. Thus earlier workers have either purified a proteolytic fraction from culture broth and inferred it to have a role in toxicity, via analogy with proteases produced by other insect pathogens (Ong & Chang, 1997; Schmidt et al., 1988; Yamanaka et al., 1992), or they have examined the toxicity of cell-free culture supernatants to insects via injection and then suggested that toxicity is correlated with the protease activity also observed in the culture broth (Jarosz et al., 1991). In our earlier work on protease purification from strain W14 we have demonstrated that proteolytic activity in the supernatant is independent of the high-molecular-mass insecticidal toxin complexes (Bowen et al., 2000). We have also shown that protease activity can be resolved into three fractions, one of ~55 kDa and two of ~40 kDa (Bowen et al., 2000). To further clarify the likely role of secreted protease, here we describe the biochemistry of the purified PrtA protein (corresponding to the ~55 kDa fraction) and the cloning of the associated prtA gene locus from two different Photorhabdus strains.

**METHODS**

**Bacterial strains and culture.** Stock cultures of strains W14 and K122 were maintained on 2 % Proteose Peptone number 3 (PP3) or Luria–Bertani (LB) agar plates, respectively. Plates were stored at 18–20°C for up to 2 months and then transferred to fresh PP3 agar. Chromosomal DNA preparations were made from overnight cultures of W14 and K122 grown in Graces insect tissue culture medium (Life Technologies) or LB liquid medium (Difco), respectively, at 29°C. Escherichia coli XL-1 Blue MRF’ (Δ(mcrA)183 Δ(mcrCB-hsdSMR-mcrA)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F’proAB lacP2ΔM15 Tn10 (Tet’) AmyC’] [Strategene]) and DH5α (F’[F’proAB lacP2ΔM15 Tn10 (Tet’) AmyC’] [Strategene] and DH5α (F’[F’proAB lacP2ΔM15 Tn10 (Tet’) AmyC’] [Strategene] and DH5α (F’[F’proAB lacP2ΔM15 Tn10 (Tet’) AmyC’] [Strategene] and DH5α (F’[F’proAB lacP2ΔM15 Tn10 (Tet’) AmyC’] [Strategene] and DH5α (F’[F’proAB lacP2ΔM15 Tn10 (Tet’) AmyC’] & ΔlacZΔM15 U169 deoR recA1 endA1 hsdR17(rK-, mK-) phaA supE44 Δ thi-1 gyrA96 relA1) (Life Technologies) were the host strains used for W14 and K122 library constructions, respectively. Genomic libraries were plated on gelatin agar containing 2 g nutrient broth (Difco) with 8 g gelatin (Difco) and 16 g agar (Difco) dissolved in 1 l distilled water.

**Protein analysis and protease purification.** Proteins were analysed by SDS-PAGE and protease activity was assessed by electrophoresis enzymograms, as described previously (Bowen et al., 2000). Culture supernatant fractions were prepared by centrifuging bacterial cultures at 14 000 g and 4°C for 5 min and precipitating supernatant proteins with 10 % trichloroacetic acid (TCA) for 30 min on ice. Precipitated proteins were pelleted at 14 000 g and 4°C for 30 min and washed once with 500 μl cold (~20°C) 80 % acetone, followed by resuspension in SDS-PAGE sample buffer and analysis by SDS-PAGE and enzymography. Protease activity was measured as the increase of BODIPY-FL fluorescence released from cleaved BODIPY-FL-labelled casein using the EnzChek Protease Assay kit (Molecular Probes). Measurements were made in a BioLum microplate fluorescence spectrophotometer (Molecular Dynamics) with excitation and emission wavelengths of 505 and 513 nm, respectively. Purified protease in 20 mM Tris/HCl, 5 mM CaCl₂, pH 8.0, was assayed in a volume of 200 μl with BODIPY-FL casein at a final concentration of 5 μg ml−1. Analysis of protease autolysis was performed in 20 mM Tris/HCl with the addition of either 10 mM CaCl₂ or 10 mM EDTA (pH 8.0) at either 30, 37, 42 or 60°C at a protein concentration of 1 mg purified PrtA ml−1. Aliquots were removed at time intervals and analysed by SDS-PAGE. Estimation of degradation half-life was made from the densitometric analysis of the peak surface area of intact mature 55 kDa protease.

Protease purification was performed as described previously (Valens et al., 2002). The cytotoxicity of purified PrtA was tested against a tissue culture of mammalian (COS7) cells by adding serial dilutions of protease to the growth medium and incubating overnight. Cells were then fixed and stained with FITC-labelled phalloidin to examine the integrity of the cells and their actin cytoskeleton.

**Insect infection and in vivo analysis of protease activity.** Fifth instar larvae of the Greater wax moth (Galleria mellonella) (Mr G. Dumond, Earl La Teigne Dorée, France) were infected intraperitoneally with 100 Photorhabdus luminescens cells (counted by plating c.f.u.) in sterile PBS. Larvae were maintained at 28°C during subsequent infection. Larval extracts were prepared at intervals by homogenizing single larvae in 1 ml PBS in a hand-held 10 ml teflon homogenizer. Tissue debris was removed by centrifugation at 2000 g and 4°C for 5 min. Supernatants were precipitated with 10 % TCA for 30 min and then centrifuged at 14 000 g and 4°C for 15 min. Precipitated pellets were washed once with 500 μl cold (~20°C) 80 % acetone and resuspended in SDS-PAGE sample buffer prior to analysis by SDS-PAGE enzymography.

**Library construction and screening.** Approximately 150 μg strain W14 phase variant 1 genomic DNA, partially digested with Sau3A, was size-fractionated on a NaCl density gradient (a linear gradient from 1:25 to 5 M NaCl in Tris/EDTA). The gradient was generated using a Bio-Rad EconoSystem, programming the pump to generate the gradient and pumping it into a 4 ml centrifuge tube. Centrifugation for 3.5 h at 44 000 r.p.m. in an SW 60 swinging bucket rotor at 18°C and were fractionated by puncturing the bottom of the tube and collecting 100 μl fractions (35–40 fractions). DNA was precipitated by adding ethanol to a final concentration of 66 %. The precipitated DNA was dissolved in 10 μl sterile water and 1 μl from every fraction was analysed by agarose gel electrophoresis to check size and concentration. Fragments larger than ~12 kb were ligated into pBluescript K5+ (Strategene) and transformed into library component E. coli XL-1 Blue MRF’ cells (Strategene). The cells were allowed to recover for 1 h in LB at 30°C and were then plated on gelatin agar with 100 μg ampicillin ml−1.
Similarly, a library of strain K122 variant I genomic DNA was constructed in BamHI-digested, dephosphorylated pUC18 (Amersham-Pharmacia Biotech) comprising fragments of between 10 and 15 kb isolated following sucrose density-gradient centrifugation of Sau3AI partially digested chromosomal DNA as above. Recombinant clones were isolated following chemical transformation of E. coli DH5α library competent cells (Life Technologies) and selection on LB agar containing 100 μg ampicillin ml⁻¹. The three positive clones secreting protease activity from the W14 genomic library were end-sequenced and their relative degree of overlap was determined by restriction mapping. Clone pPRT1-W14 was chosen since it was the smallest clone still retaining secreted proteolytic activity. Deletions, based upon suitable restriction sites, were then made within pPRT1-W14 both for further nucleotide sequencing and also for re-assay for protease activity on gelatin agar plates. The sequence of both strands of the complete W14 protease operon, and its associated Type I transporter, was finished by primer walking.

RESULTS

Cloning and analysis of the prt operon

Screening of approximately 5000 recombinant E. coli XL-1 Blue MRF colonies from the W14 library resulted in the identification of three overlapping protease-positive clones (Fig. 1a). Screening of approximately 20 000 colonies from the K122 library did not reveal any positive clones in E. coli DH5α recombinants. The nucleotide sequence of pPRT1-W14 predicts an operon of four ORFs (Table 1) all transcribed in the same direction (Fig. 1b). The first is the structural gene for the protease, prtA, followed by a gene encoding a putative protease inhibitor, inh (Valens et al., 2002) and then the three members of an associated Type I transporter, prtB, prtC and prtD. We have therefore cloned all the genes necessary for the secretion of PrtA from recombinant E. coli.

The K122 operon was isolated following screening of the K122 library with a radioactive [³²P]dCTP-labelled probe containing a PCR product of the W14 prtA gene. Three positive clones were identified from a screen of approximately 10 000 E. coli DH5α recombinants. One clone, pPRT-K122, was selected and the DNA sequence was determined for the 5’ and 3’ ends of the prtA gene. The insert of pPRT-K122 is ~12 kb comprising the entire prt operon and 1798 bp upstream of prtA. This upstream region contains a truncated ORF (minus the first 197 bp) with homology to E. coli ygdE, encoding a hypothetical protein in the fucR-gcvA intergenic region. GcvA is a member of the LysR transcriptional regulator family (Everett et al., 1995). The

![Fig. 1. Diagram of the prt operon. (a) Relative locations of the clones from strains W14 and K122. (b) Organization of the W14 prt locus and its associated ABC transporter. The locus contains the structural gene for the protease (prtA), followed by its putative inhibitor (inh) and then the associated ABC transporter (prtBCD). (c) Deletion analysis of the prt operon shows that loss of either part of the prtA structural gene or part of the terminal ABC transporter ORF (prtD) results in loss of recombinant secretion of PrtA by E. coli. The presence (+) or absence (−) of recombinant proteolysis (zone of clearing around colony on gelatin agar) for each plasmid is shown.](http://mic.sgmjournals.org)

http://mic.sgmjournals.org
Table 1. Characteristics of the ORFs and predicted proteins from the prtA1 operon of strain W14

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of amino acids</th>
<th>Calculated mass (Da)</th>
<th>Calculated no. of TMD*</th>
<th>Closest homologue†</th>
<th>Organism†</th>
<th>Similarity (identity)‡</th>
<th>Accession no.†</th>
</tr>
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<tbody>
<tr>
<td>prtA1</td>
<td>486</td>
<td>53 860</td>
<td>0</td>
<td>AprA</td>
<td>Ps. aeruginosa</td>
<td>66 (52)</td>
<td>Q03023</td>
</tr>
<tr>
<td>prtI</td>
<td>134 (108)§</td>
<td>14 795</td>
<td>0</td>
<td>Inh</td>
<td>Erw. chrysanthemi</td>
<td>55 (46)</td>
<td>P18958</td>
</tr>
<tr>
<td>prtB</td>
<td>547</td>
<td>59 493</td>
<td>6</td>
<td>AprD</td>
<td>Ps. aeruginosa</td>
<td>72 (56)</td>
<td>Q03024</td>
</tr>
<tr>
<td>prtC</td>
<td>442</td>
<td>49 006</td>
<td>1</td>
<td>AprE</td>
<td>Ps. aeruginosa</td>
<td>64 (46)</td>
<td>Q03025</td>
</tr>
<tr>
<td>prtD</td>
<td>457</td>
<td>51 957</td>
<td>1</td>
<td>AprF</td>
<td>Ps. aeruginosa</td>
<td>63 (45)</td>
<td>Q03027</td>
</tr>
</tbody>
</table>

*Transmembrane domains calculated using the program TopredII (Spencer et al., 2000).
†The corresponding protein, organism and SWISS-PROT database accession numbers are given for the closest homologues identified by a BLAST search.
‡Percentage identity and similarity calculated from BLAST.
§The number in parentheses indicates the mature protein following N-terminal signal sequence cleavage.

pPRT1-W14 clone insert has a length of 7399 bp starting at position −845 with respect to the initiation codon of prtA and is missing the ygdE-like ORF.

The predicted PrtA proteins from the two different strains are 87-6% identical and 92-1% similar. The predicted amino acid sequence of PrtA confirms that it is an RTX-like zinc metalloprotease similar to proteases from Erwinia chrysanthemi and Serratia marcescens. Alignments (Table 1) show the conservation of the RTX repeats with the Prosite motif D-x-[LI]-x(4)-G-x-d-[LI]-x-G-x(3)-D (Prosite Motif PDOC00293) implicated in Ca²⁺ binding and a putative zinc-binding motif (position 184–193, TTFHE IGHTL) with the consensus sequence [GSTALIVN]-x(2)-H-E-[LIVMFYW]-x-LIVMFYWSPQ (Prosite Motif PDOC00129). The protease inhibitor is most similar to the inh gene of Erwinia chrysanthemi and is predicted to possess an N-terminal secretion signal (cleavage between residues 26–27) consistent with export to the periplasm via the sec-dependent pathway, as confirmed previously (Valens et al., 2002). The members of the putative Type I trans-envelope transporter are most similar to the apr genes of the Pseudomonas aeruginosa RTX-like zinc metalloprotease transporter and include an ATP-binding cassette (ABC) protein, PrtB, a membrane-fusion protein (MFP), PrtC and an outer-membrane protein (OMP), PrtD. A series of six internal deletions within the W14 prt operon on plasmid pPRT1-W14 were obtained using internal restriction endonuclease cleavage sites (Fig. 1c) and all six clones were defective for the secretion of active protease. This indicates that all of the genes in the prt operon are required for the synthesis and secretion of PrtA to the culture medium.

Analysis of PrtA secretion from Photorhabdus luminescens and recombinant E. coli

Photorhabdus strains exhibit ‘phase variation’ whereby individual strains show phenotypic variation between phase I and phase II which differ in colony colour and morphology. To examine possible differences in expression between these variants, phase I and II colonies, from both strains W14 and K122, together with E. coli DH5α harbouring either plasmid pPRT-K122 or pPRT1-W14 were grown to stationary phase in LB medium at 30°C with agitation. Culture supernatants were prepared and analysed by SDS-PAGE by both Coomassie blue staining (Fig. 2a) and gelatin immunoblot (Fig. 2b). The Coomassie-blue-stained profile shows large numbers of proteins in the medium, secreted by both phase I and II variants of both strains W14 and K122 (Fig. 2a, lanes 1, 3, 5 and 6). Both W14 and K122 phase I cells secrete qualitatively and quantitatively different profiles (lanes 1 and 3) and this difference is also reflected between the phase II variant cells (lanes 5 and 6). Moreover, in both strains, phase I cells secrete considerably more proteins than the phase II cells. The entire prt operon of W14 and K122 is present on recombinant clones pPRT1-W14 and pPRT-K122, including 845 and 1798 bp of upstream DNA sequence, respectively. However, secretion of a protein with an apparent molecular mass of 55 kDa from E. coli DH5α harbouring pPRT1-W14 is clearly evident (lane 4), whereas no abundant 55 kDa band is present in the supernatant from E. coli DH5α harbouring pPRT-K122. Protease activity is associated with a minor 55 kDa band in supernatants of pPRT-K122 (lane 2), indicating that, unlike the W14 clone pPRT-W14, the K122 operon harboured in pPRT-K122 secretes substantially less protease to the supernatant when expressed in E. coli DH5α.

Protease purification and activity

We purified native PrtA from strain K122 as this strain produces less extracellular polysaccharide than W14, thus facilitating purification of PrtA directly from the supernatant. (We recovered recombinant PrtA-expressing clones from strain W14 as clones from this strain produced more protease and thus could be detected in library screens, whereas clones from K122 libraries could not.) For purification, culture supernatants from K122 phase I cells were prepared as described above and the PrtA protease activity was enriched through Octyl Sepharose Fast Flow hydrophobic interaction chromatography. Protease-containing...
fractions were pooled and further purified by MonoQ cation-exchange chromatography. Peak fractions containing the purified protease were analysed by SDS-PAGE and Coomassie blue staining (Fig. 3) and revealed a single band migrating at 55 kDa of purity >95% and a yield of up to 25 mg (l culture). PrtA protease activity against BODIPY-FL-labelled casein was examined (Fig. 4). Activity against this artificial substrate was high with detectable hydrolysis after the addition of as little as 0.9 pM enzyme and strong initial rates of reaction. Kinetic parameters cannot be readily determined for a complex, multi-cleaved substrate such as casein; however, a clear dependence of initial hydrolysis rate ($V_i$) against enzyme concentration could be shown (graph inset, Fig. 4). An interesting difference in enzyme activity compared to the Protease II secreted by *Xenorhabdus nematophila* (Caldas *et al.*. 2002), also an RTX zinc metalloprotease, is that the range of artificial substrates employed by the latter authors were not cleaved by PrtA. To date, we have not identified a cleavable colorimetric artificial substrate permitting a Michaelis–Menten analysis of protease activity.
Inhibition of PrtA by protease inhibitors

To characterize the biochemistry of the purified protease we tested residual activity after the addition of a range of diagnostic protease inhibitors (Fig. 5). Little inhibition was observed with PMSF, PCMB and pepstatin which are inhibitors of serine, thiol and aspartate/acid proteases, respectively. However, significant levels of inhibition were observed following incubation with either EDTA (a general metalloprotease inhibitor) or 1,10-phenanthroline (a specific zinc metalloprotease inhibitor). These data are consistent with the hypothesis that PrtA is a zinc metalloprotease. The apparent molecular mass and zinc dependence of PrtA suggest that this enzyme is of the same family of metzincin metalloendopeptidases belonging to the RTX family. Interestingly, K122 PrtA was inhibited by a metalloprotease inhibitor; filled squares, PMSF, a serine protease inhibitor; open squares, PCMB, a thiol protease inhibitor; filled triangles, pepstatin, an aspartate and acid protease inhibitor.

Autocleavage of the PrtA protease

RTX proteins are characterized by multiple glycine-rich nonapeptide repeats within the C terminus involved in calcium ion binding (Welch, 1991). The purified PrtA protease was tested for autocatalytic cleavage activity in the presence or absence of Ca^{2+} ions (Fig. 6). In the presence of additional 10 mM CaCl_2, PrtA undergoes a time-dependent autocleavage at 42 °C, resulting in three major peptide fragments (Fig. 6a). The presence of 10 mM EDTA results in a greater number of cleavage products (Fig. 6b). The half-life of autocleavage, estimated following densitometric analysis of stained bands, under both conditions remains the same (Fig. 6c), indicating that although the presence or absence of Ca^{2+} affects the autocleavage profile, it does not alter the rate of degradation. Furthermore, EDTA effectively inhibits PrtA activity against a foreign substrate, but does not affect internal autocleavage.

Although Photobacterium luminescens is a psychrophile with an optimum growth temperature of 28 °C, the PrtA protease is remarkably thermostable. Fig. 7 illustrates the half-life of time-dependent autocleavage of purified PrtA with increasing temperature. At 30 and 37 °C little degradation is observed with a high stability at the more physiological temperature of 30 °C (half-life > 48 h). Incubation at 42 and 60 °C show increased rates of degradation with half-lives of 52.1 ± 3.5 and 9.1 ± 1.8 min, respectively. Together with the Ca^{2+}-dependent autocleavage profiles (Fig. 6), this suggests that Ca^{2+} ions play an important role in structural stability, but not in enzymic activity per se.

Appearance of protease activity during insect infection

Purified K122 PrtA protease and supernatants from both E. coli DH5α expressing either pPRT-W14 or pPRT-K122, were not toxic to Manduca sexta third instar or G. mellonella fifth instar larvae with up to 10 μg PrtA, suggesting that PrtA does not have direct toxicity to insects. To confirm that PrtA is actively expressed during an insect infection we used the enzymogram technique to monitor the presence of active protease in G. mellonella larvae infected with either phase I or phase II bacteria from strain K122. Following an in vivo infection by ventral injection directly into the haemocoel with 1000 phase I bacteria, active protease was observed in total protein extracts from insect homogenates as early as 28 h after injection (Fig. 8). As this protein migrates at ~55 kDa on an enzymogram, we are confident that this represents PrtA activity. This protease activity was still present at 48 h post infection. In contrast infections of phase II bacteria failed to show active protease at 24 h, but did show equivalent levels of activity 48 h post-infection, suggesting that phase II cells still express protA but at lower levels. The sensitivity of detection was estimated to be between approximately 10 and 100 pg PrtA from analysis of purified enzyme on the same enzymogram. Previous studies of protease activities from W14 cultures suggest that PrtA is secreted as the bacteria enter stationary phase (Daborn et al., 2001). Absence of detectable PrtA activity on enzymograms prior to late infection is unlikely to be due to inhibition of PrtA by an insect inhibitor since enzymograms are performed upon insect extracts precipitated with 10% TCA and migrated with SDS and β-mercaptoethanol. PrtA is capable of renaturing following migration and experiments performed by prior incubation of PrtA with its cognate
inhibitor Inh (Valens et al., 2002) indicate that prior inhibition of PrtA is alleviated following migration in SDS-PAGE, due to the dissociation of the inhibitor. However, we cannot formally exclude the possibility of covalent inhibition by an insect factor.

**Effects of PrtA on mammalian cells**

COS7 cells were grown overnight to a confluent monolayer before application of the protease. After incubation overnight with dilutions of the purified protease (Fig. 9) controls lacking PrtA remained as intact confluent cell layers whose actin cytoskeleton is clearly visible and intact. After exposure to as low a concentration as 0·05 μg PrtA ml⁻¹ the cell–cell contacts within the monolayer are broken and actin condensation can be seen in some cells. At higher concentrations of PrtA (0·5 μg ml⁻¹) the cells begin to break apart and detach until at 5·0 μg ml⁻¹ most of the cells have left the cover slip before fixation. These results are consistent with PrtA having a directly cytotoxic effect on these cells.

**DISCUSSION**

Few of the numerous proteins and candidate virulence factors secreted by *Photorhabdus* have been characterized in detail (ffrench-Constant et al., 2003). Since many extracellular bacterial proteases have either been demonstrated or suggested to play a role in virulence, here we have investigated the dominant proteolytic activity associated with culture supernatants of *Photorhabdus* via the cloning and characterization of the *prtA* locus. Our previous work on protease purification from strain W14 showed that
protease activity can be clearly separated from the oral activity of the toxin complexes (Bowen et al., 2000). A further three distinct protease fractions were purified, one of ~55 kDa and two others of ~40 kDa. The prtA locus cloned here appears to encode the dominant species in the 55 kDa fraction. The characterization of the inhibitor encoded by prtl has been described previously (Valens et al., 2002).

We purified native PrtA from strain K122, as this strain produces less extracellular polysaccharide than W14, thus facilitating purification of PrtA directly from the supernatant. We recovered recombinant PrtA-expressing clones from strain W14 as clones from this strain produced more protease and thus could be detected in library screens, whereas clones from K122 libraries could not. The genomic organization of the prtA locus from strain W14, with the structural gene for the metalloprotease and its associated type I transporter being found together, differs from that seen in other bacteria. Only one protease-encoding ORF, prtl, is associated with the operon, unlike that of Erwinia chrysanthemi B374 which has four associated protease-encoding genes (Delepelaire & Wandersman, 1990; Ghigo & Wandersman, 1992a, b). The prtA gene is termed prtl as the available genomic sequence predicts a homologue of prtl (prtA2) elsewhere in the W14 genome. The prt operon architecture in strain W14 resembles most closely that of the Pseudomonas aeruginosa apr operon. Indeed, BLAST analysis of the SWISS-PROT database indicates that the Photorhabdus luminescens protease, ABC transporter, MFP and OMP are most closely related at the amino acid sequence level to the Apr functional homologues. The exception is that of the gene encoding the inhibitor, inh, which is most closely related to that from Erwinia chrysanthemi.

Purification of native PrtA protease from strain K122 was achieved directly from stationary-phase culture supernatants. The protease is remarkably stable and resistant to high temperature, detergent and the presence of reducing agents. Biochemical analysis using protease inhibitors indicated that the enzyme is a zinc-dependent alkaline metalloprotease and autocatalytic cleavage peptide profiles in the presence and absence of calcium suggested that PrtA requires bound calcium to stabilize its structure, as for other RTX proteins. Both primary- and secondary-phase variants secrete PrtA to the medium as detected by enzymography. However, direct measurements of PrtA activity in culture supernatants of both K122 and W14 indicate that the actual secreted native activity from phase II cells is approximately 25-fold lower than that of phase I cells (data not shown). Such a difference is not as manifest in SDS-PAGE enzymograms. This may be due to an activation of inherently inhibited phase II secreted protease following treatment with SDS, reducing agent and heating. Previous studies have suggested that primary-phase variants of Photorhabdus temperata produce more active lipase enzyme than the secondary variants, whilst levels of lipase gene transcription remain the same (Wang & Dowds, 1993). This led to the hypothesis that the difference in the specific activity of the lipase between the two phenotypic variants may be associated with post-translational regulation (Wang & Dowds, 1993). Whilst we did not examine levels of PrtA gene transcription in the two variants, we note that differences in the association of PrtA with its candidate inhibitor represent

![Fig. 7. PrtA protease autolysis is temperature-dependent. Purified protease was incubated in 10 mM calcium chloride at 30°C (filled circles), 37°C (open circles), 42°C (filled squares) and 60°C (open squares) and the autolysis of the mature 55 kDa species was measured by band densitometry. Half-lives (± SEM) of three replicates were determined to be >48 h, >48 h, 52.1 ± 3.5 min and 91 ± 1.8 min at 30, 37, 42 and 60°C respectively.](image)

![Fig. 8. PrtA activity during an in vivo insect infection. G. mellonella insect larvae were infected with 100 phase I or II K122 cells in PBS. Whole larvae were homogenized in 1 ml PBS at the times indicated post-infection and aliquots of soluble protein were analysed by an SDS-PAGE enzymogram. Purified protease (1, 0.1 and 0.01 ng) was migrated as a control for detection sensitivity.](image)
a potential mechanism for generating the apparent differences in expression we observed in this study. In this respect, a recent report describes the secretion of a broad-spectrum protease inhibitor from *Photorhabdus luminescens* isolated from *Heterorhabditis bacteriophora* HP88 nematodes (Wee et al., 2000). The authors describe the purification of a 12 kDa protein, secreted into the medium by phase II variant cells, able to inhibit endogenous secreted *Photorhabdus luminescens* protease activity. The small size of HP88 Inh is similar to that of the predicted mature size of the K122 and W14 Inh protein of 11 kDa. The K122 and W14 Inh is predicted to possess a classical sec pathway-dependent N-terminal secretion signal, and is targeted to the periplasm in both phase I and II cells (Valens et al., 2002). Although the N-terminal amino acid sequence of HP88 Inh (STGIVTFKNDXGEDIV) (Wee et al., 2000) is not similar to the predicted mature N terminus of K122 and W14 Inh (SSLVLPHELKGVWQL), we cannot exclude the possibility that they are of similar origin and function. If phase II variant cells release Inh to the medium from the

![Fig. 9. Cytotoxicity of PrtA to mammalian tissue culture cells. Effect of differing concentrations of purified PrtA added to a monolayer of COS7 cells. Panel (a) shows a confluent monolayer of cells with intact actin cytoskeletons (control). At low protease concentrations (b, 0.05 µg ml⁻¹; c, 0.5 µg ml⁻¹) the cells are beginning to pull away from each other and some cells begin to show actin condensation. At high concentrations (d, 5 µg ml⁻¹) most of the cells have already detached from the coverslip before fixation and those that remain have highly condensed actin cytoskeletons.](http://mic.sgmjournals.org)
periplasm then we would predict that it would inhibit secreted PrtA and thus lead to the observation of reduced levels of specific PrtA activity in phase II culture supernatants as compared to those of phase I cells. Moreover, it is also likely that such an interaction would be disrupted upon SDS-PAGE, thus permitting the detection of elevated levels of PrtA activity in enzymograms as observed in this and earlier reports (Wee et al., 2000).

Analysis of protease activity in G. mellonella insect larvae infected with phase I or phase II K122 cells reveals that a protease species of the correct size for PrtA is detectable late in insect infection and shortly prior to larval death. Moreover, active enzyme is produced by the phase II variant cells much later than the phase I variant. Both phase I and II variants are equally virulent to G. mellonella larvae with death occurring at approximately 24 h post-infection in both cases. Combined with the observation that injected protease is not directly toxic to G. mellonella, this suggests that PrtA is not a major virulence factor, but that it may have an alternative role in host bioconversion. Purified PrtA did however show detrimental effects on mammalian cells at low concentrations, although we noted that more non-specific proteases such as trypsin may promote a similar effect. We also noted that earlier immunocytochemistry studies with an anti-PrtA antibody have show PrtA immunoreactivity associated with the basal lamina of tissues within the insect, suggesting that the protease may attack these membranes that surround individual organs within the insect (Silva et al., 2002).

In summary, here we have described the biochemical characterization of a Photobacterium extracellular protease, PrtA, and its associated Type I secretion apparatus. PrtA is a zinc metalloprotease of the RTX family and is encoded by the prtA gene which lies within a locus also encoding its own inhibitor and associated ABC transporter. Study of the expression of PrtA within infected insects supports its suggested role in host bioconversion as the active enzyme appears in the infected cadaver after the insect has already been killed (Daborn et al., 2001).

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