The AprV5 Subtilase Is Required for the Optimal Processing of All Three Extracellular Serine Proteases from *Dichelobacter nodosus*

Xiaoyan Han1,2, Ruth M. Kennan1,2, David L. Steer1,3, A. Ian Smith1,3, James C. Whisstock1,3, Julian I. Rood1,2*

1 Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, Victoria, Australia, 2 Department of Microbiology, Monash University, Clayton, Victoria, Australia, 3 Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia

**Abstract**

*Dichelobacter nodosus* is the principal causative agent of ovine footrot and its extracellular proteases are major virulence factors. Virulent isolates of *D. nodosus* secrete three subtilisin-like serine proteases: AprV2, AprV5 and BprV. These enzymes are each synthesized as precursor molecules that include a signal (pre-) peptide, a pro-peptide and a C-terminal extension, which are processed to produce the mature active forms. The function of the C-terminal regions of these proteases and the mechanism of protease processing and secretion are unknown. AprV5 contributes to most of the protease activity secreted by *D. nodosus*. To understand the role of the C-terminal extension of AprV5, we constructed a series of C-terminal-deletion mutants in *D. nodosus* by allelic exchange. The proteases present in the resultant mutants and their complemented derivatives were examined by protease zymogram analysis, western blotting and mass spectrometry. The results showed that the C-terminal region of AprV5 is required for the normal expression of protease activity, deletion of this region led to a delay in the processing of these enzymes. *D. nodosus* is an unusual bacterium in that it produces three closely related extracellular serine proteases. We have now shown that one of these enzymes, AprV5, is responsible for its own maturation, and for the optimal cleavage of AprV2 and BprV, to their mature active forms. These studies have increased our understanding of how this important pathogen processes these virulence-associated extracellular proteases and secretes them into its external environment.

**Introduction**

*Dichelobacter nodosus* is a slow growing gram-negative anaerobic bacterium that is the principal causative agent of footrot in ruminants. Ovine footrot is characterized by the separation of the keratinous hoof from the underlying tissue, leading to lameness, loss of body weight, and reduced wool growth and quality, which results in significant economic losses [1]. Clinical disease is dependent upon the virulence properties of the *D. nodosus* isolate and the presence of warm wet climatic conditions. There are two major clinical forms of footrot, benign and virulent, with benign footrot presenting as an interdigital dermatitis with no further disease progression whilst virulent disease leads to a severe under-running of the horn of the hoof. The virulence factors of *D. nodosus* include its extracellular subtilisin-like serine proteases (or subtilases) [2–6], type IV fimbriae [7,8] and potentially, the *vrl* and *vap* genomic islands, which are preferentially associated with virulent strains [9–12].

Subtilases are produced by a wide variety of archaea, bacteria, fungi and eukaryotes. They mostly are synthesized as pre-pro-enzyme precursors that are subsequently translocated across the cell membrane and then activated by cleavage of the 27–280 residue pro-domains [13]. The relatively conserved catalytic domains contain 268–511 residues, with some members of the subtilase family also having C-terminal extensions with variable lengths and less sequence conservation [13]. The function of the C-terminal extensions is also variable. Studies on aqualysin I from *Thermus aquaticus* YT-1 [14], the serine protease SSP from *Serratia marcescens* [15] and the IgA protease from *Neisseria gonorrhoeae* [16] showed that the C-terminal extensions of these proteins are required for extracellular secretion of the proteases, while the C-terminal extension of the subtilase SISBTS from the tomato plant is required for both pro-domain processing and secretion [17]. By contrast, it has been shown that the C-terminal extension of MCP-03, a subtilase from *Pseudoalteromonas* sp. SM9913, is not crucial for secretion, but instead negatively affects catalytic efficiency and is essential for protease thermostability [18]. Studies on the Lactococcus lactis protease suggested that some residues in the C-terminal extension affect catalytic activity [19].

Three closely related subtilases are secreted by virulent strains of *D. nodosus*: two acidic proteases, AprV2 and AprV5, and the...
basic protease BprV. The equivalent proteases in benign strains are AprB2, AprB5 and BprB [20,21]. AprV5 has been shown to be responsible for most of the extracellular protease activity in a virulent strain of D. nodosus, VCS1703A, although AprV2 is responsible for most of the extracellular elastase activity and is essential for virulence [6]. These proteases are encoded by separate genes and are synthesized as precursors with an N-terminal pre-pro-region, a serine protease domain and a C-terminal extension. Based on the cleavage sites determined previously [20,21], the estimated sizes of the mature domains of AprV5, AprV2 and BprV are 36.0 kDa, 36.4 kDa and 35.8 kDa, respectively; their respective C-terminal extensions are 13.9 kDa, 14.0 kDa and 14.1 kDa in size. The amino acid sequences and structures of the catalytic protease domains are highly conserved (~65% identity) [6,22], but the sequences of the C-terminal extensions are less conserved, with only approximately 35% similarity. The C-terminal extension of AprV5 contains a P-domain, which is typically associated with eukaryotic pro-protein convertases that belong to the subtilisin-like superfamily [13,23]. The active proteases are produced by cleavage of the pre-pro region and the C-terminal extension [2,24,25]. The mechanism of protease processing and secretion is unclear, although type IV fimbriae are required for optimal secretion [7,8,26].

In this study, we aimed to examine the mechanism of processing of the extracellular proteases from D. nodosus and to determine the role of their C-terminal extensions. To achieve these aims we constructed a series of C-terminal truncated aprV5 mutants in D. nodosus by allelic exchange. The results showed that AprV5 was required for efficient processing of AprV2 and BprV and that the C-terminal extension of AprV5 was required for maximal AprV5 activity.

Materials and Methods

Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids are listed in Table 1. Escherichia coli DH5α and NovaBlue cells used for plasmid propagation and cloning experiments were grown at 37°C on 2xYT medium [27].

The transformable virulent D. nodosus strain VCS1703A and its derivatives were grown in an Anaerobic Chamber (Coy Laboratory Products Inc.) in an atmosphere of 10% (vol/vol) H2, 10% (vol/vol) CO2, and 80% (vol/vol) N2 on Egon (BBL) yeast extract (EYE) agar with 5% (vol/vol) defibrinated horse blood (Bio-lab) or in TASS broth [28], as described previously [7,26]. When required, media were supplemented with the following antibiotics at the indicated concentrations: for E. coli, ampicillin (100 µg/ml), kanamycin (20 µg/ml) or erythromycin (150 µg/ml), and for D. nodosus, ampicillin (10 µg/ml), kanamycin (10 µg/ml) or erythromycin (1 µg/ml).

DNA Manipulation and Molecular Techniques

Unless otherwise stated, standard procedures were used [27]. D. nodosus genomic DNA was prepared using a QIAGEN DNeasy kit, according to the manufacturer’s instructions. Southern hybridizations were performed as described previously [26,29], with probes specific for the relevant antibiotic resistance genes or the target genes. Reverse transcriptase (RT)-PCR was carried out as previously described [8]. DNA sequencing was performed using an Applied Biosystems 3730S Genetic Analysers. Sequence data were compiled using Sequencher version 3.0 (Gene Codes Corporation) or Vector NTI advance™ 11 (Invitrogen).

Construction of Suicide Vectors and Mutants of D. nodosus

All of the suicide vectors used in this study were constructed in pUC18K [30], which does not replicate in D. nodosus. They contained two 1.2-kb to 1.9-kb D. nodosus-derived DNA fragments that were located on either side of an aphA-3 kanamycin-resistance cassette, with a bla ampicillin-resistance gene located on the plasmid vector. Therefore, D. nodosus transformants that were derived from double crossovers could be obtained by selecting for kanamycin resistance and screening for susceptibility to ampicillin.

To construct an aprV5Δ5478–595 mutant, the suicide vector pJR3520 was constructed as follows. A 1.5-kb PCR product that contained the aprV5 gene region that encoded AprV5 residues 1–477 was cloned into the SacI/KpnI sites of pUC18K followed by the cloning of a 1.5-kb PCR fragment located downstream of aprV5 into the BamHI/XbaI sites of the resultant plasmid.

To construct the aprV5Δ503–595 suicide vector pJR3708, the 1.5-kb aprV5Δ5478–595 fragment of pJR3520 was deleted by SacI/KpnI digestion and replaced with a 1.4-kb PCR product containing aprV5Δ503–595. The aprV5Δ503–595 suicide vector pJR3729 was constructed in a similar way. It contained the 1.5-kb aprV5 downstream region inserted into BamHI/XbaI sites and a 1.8-kb fragment containing aprV5Δ503–595 that was obtained by EcoRI/SacI digestion from pJR3695, a plasmid containing a full-length aprV5 gene cloned into the KpnI site of pGEM7z(+). Finally, an aprV5His6 vector (pJR3661) was constructed in a similar manner by cloning a 1.2-kb PCR product containing an N-terminal truncated aprV5 plus a hexahistidine-encoding sequence into the KpnI site and cloning a 1.2-kb PCR product downstream of aprV5 into the XbaI/PstI sites of pUC18K.

To complement the truncated aprV5 mutants, a 1.2-kb PCR product from the region downstream of aprV5 was cloned into the XbaI/PstI sites of pUC18K and then a 1.9-kb fragment containing the region encoding AprV5 C-terminal extension was cloned into the KpnI site of the resultant plasmid to form pJR3711. To enable an antibiotic selection for the complemented strains, aphA-3 was deleted from pJR3711 by SmaI digestion and replaced by an emrB cassette from pJR2412 [26] to form the complementation vector pJR3730. The complemented strains were selected on erythromycin and screened for susceptibility to kanamycin and ampicillin.

Plasmid DNA was introduced into D. nodosus by natural transformation and mutants were constructed by homologous recombination between the double crossover suicide vectors and the D. nodosus chromosome, as described previously [7,8]. Transformants were initially screened by their resistance or susceptibility to the appropriate antibiotics as described above. Potential mutants were analysed by PCR using relevant antibiotic-resistance gene and target gene primers. Southern hybridization was used to confirm that the C-terminal extension encoding region had been deleted or that a 6xHis-encoding tag had been incorporated onto the C-terminus of aprV5 by double crossover events.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Whole cell extracts of D. nodosus strains were prepared and culture supernatants were trichloroacetic acid (TCA)-precipitated as previously described [8]. Proteins were detected by SDS-PAGE on 4–15% gradient gels (BioRad) after staining with Coomassie Brilliant Blue. The proteases AprV5 and BprV were analysed by immunoblotting with a 1:4000 diluted AprV5- and BprV-specific antiserum raised in sheep (O. Dhungyel & R. Whittington,
University of Sydney), respectively. AprV2 was immunoblotted with a 1:300 dilution of AprV2-specific rabbit antisera (Millipore).

Extracellular Protease Assays

Caseinase activity was detected qualitatively by growing nodosus colonies on EYE agar containing 2% (wt/vol) skim milk [31]. Total protease activity in the culture supernatant was determined quantitatively using azocasein (Sigma) as the substrate, as previously described [7]. One unit of protease activity is defined as previously described [7].

Mass Spectrometry and Protein Identification

Protein bands of interest were excised from Coomassie Brilliant Blue stained gels and prepared for MS analysis as described previously [33]. After the washing and dehydration steps the gel pieces were rehydrated with a solution containing 0.5 μg of sequencing grade trypsin, incubated at 37°C overnight and sonicated. Digested samples were co-spotted onto the target plate, overlaid with gelatin/agarose and incubated for 75 min at 37°C. The gel was washed in tap water prior to photography.
Results

The C-terminal Extension of AprV5 is Required for Processing of AprV2

In studies aimed at determining the role of these extracellular proteases in virulence, we previously constructed mutations in each of the three D. nodosus extracellular protease genes [6]. To determine if mutation of any of these proteases altered the production of the other two enzymes, the extracellular protease activity of these insertional-inactivated aprV5, aprV2 and bprV mutants and their complemented derivatives was characterized by protease zymogram analysis after native gel electrophoresis. The results (Figure 1) showed that, as expected, in the aprV2 mutant, there was no AprV2 activity, as represented by the two missing isoenzyme activity bands; these bands were restored by complementation. Mutation of aprV2 had no effect on AprV5 activity (the band of highest electrophoretic mobility). By contrast, in the aprV5 mutant the AprV5 band was absent as expected and the two AprV2 bands were either not present (Figure 1), or in some preparations were very greatly reduced in intensity; all of these protease bands were restored in the complemented aprV5 derivative (Figure 1). Based on these studies we postulated that AprV5 was required for the correct processing of AprV2. Finally, there was no difference in the active AprV2 or AprV5 bands in the bprV mutant (Figure 1). However, an additional protease activity band, of slightly greater electrophoretic mobility than the faster of the AprV2 bands, was observed in this strain. This band disappeared in the bprV-complemented derivative, but although difficult to see in this specific preparation; both of the AprV2-derived protease bands were always present in supernatants from this strain. Note that the basic protease BprV can not be observed by this method, as a result of its basic pI. Attempts to visualise BprV activity by carrying out zymogram analysis under different electrophoretic conditions were unsuccessful.

To determine if the C-terminal domain of AprV5 was involved in the processing of AprV2 we again used a genetic approach. The aprV5 sequence encoding the previously determined C-terminal extension, residues 478–595 [25], was deleted by allelic exchange between the suicide vector pJIR3520 and the wild-type chromosome. The genotype of the C-terminal-truncated strain aprV5Δ478–595, which was designated as aprV5ΔC1 (Figure 2), was confirmed by PCR analysis and Southern hybridization. To ensure that the C-terminal deletion had no effect on protease gene expression, RT-PCR analysis using aprV5-, aprV2- and bprV-specific primers was performed. The results showed that aprV5, aprV2 and bprV were all transcribed in this mutant (data not shown). We subsequently constructed two additional AprV5-C-terminal truncated strains, aprV5Δ503–595 (aprV5ΔC2) and aprV5Δ560–595 (aprV5ΔC3) (Figure 2), which also were confirmed by PCR and Southern hybridization. All three mutants were complemented by using homologous recombination to reconstitute the deleted C-terminal-encoding aprV5 regions.

Qualitative protease analysis on skim-milk agar was carried out on the C-terminal deletion strains and their complemented derivatives. The results showed that after 24 h incubation, all of the aprV5ΔC mutants had reduced protease activity compared to the wild-type strain; this activity was restored in each of the complemented derivatives (data not shown). These results were confirmed by quantitative protease assays carried out on culture supernatants of 16 h (mid-logarithmic growth phase), 25 h (late logarithmic/early stationary phase) and 40 h (stationary phase) TAS broth cultures of each strain, using azocasein as the substrate. An aprV5 null mutant [6] was included as a negative control in this experiment. The 16 h and 25 h samples, which represented late logarithmic and stationary growth phases, respectively, of each of the aprV5ΔC mutants, had significantly lower levels (p<0.05, student’s t-test) of extracellular protease activity than the wild-type strain (Figure 3). There was no significant difference in activity in the 16 h culture supernatants between any of the mutants, WT (595 aa), AprV5ΔC1 (477 aa), AprV5ΔC2 (502 aa) and AprV5ΔC3 (559 aa).

Figure 1. Zymogram analysis of extracellular protease activity. Gelatin was used as a substrate overlay of supernatants separated by native polyacrylamide gel electrophoresis [32] to screen AprV5 and AprV2 activity in 25 h TAS broth cultures from the wild-type strain VCS1703A (WT), the aprV5 mutant JIR3756 (aprV5), the aprV2 mutant JIR3743 (aprV2), the bprV mutant JIR3928 (bprV) and the complemented derivatives JIR3883 (aprV5(aprV5ΔC1)), JIR3900 (aprV2(aprV2ΔC2)) and JIR3930 (bprV(bprVΔC3)), respectively. doi:10.1371/journal.pone.0047932.g001

Figure 2. Schematic representation of the C-terminal deleted AprV5 derivatives. The number of amino acid residues in each derivative is indicated as are the pre-, pro-, mature and C-terminal extension (CTE) regions. doi:10.1371/journal.pone.0047932.g002
although they all had significantly less protease activity than the wild type (p<0.05). However, the protease activity in 25 h culture supernatants of the aprV5 and aprV5ΔC1 mutants was significantly lower (p<0.05) than that of the aprV5ΔC2 and aprV5ΔC3 mutants. By contrast, at 40 h there was no significant difference between the aprV5ΔC mutants and the wild-type strain. The reduced protease activity of 16 h and 25 h cultures of each aprV5ΔC strain was restored to the wild-type level upon reconstitution (Figure 3). These results implied that deletion of the C-terminal domain of aprV5 delays the appearance of fully functional extracellular protease in the culture supernatant of D. nodosus.

To determine if the C-terminal deletions of AprV5 affected the processing of AprV2 in a similar manner to the aprV5 null mutant (Figure 1), protease zymogram analysis was carried out. The results showed that the AprV5 activity in the 16 h supernatants from each aprV5ΔC mutant was significantly reduced (Figure 4); moreover, AprV2 activity also was reduced in these samples. In addition, extra bands of lower electrophoretic mobility than AprV2 were present in these samples, presumably unprocessed forms of the proteases. At 25 h the AprV5 and AprV2 activity bands in the aprV5ΔC mutants were not discernibly different to those of the wild-type strain (Figure 4), although in the aprV5ΔC1 and aprV5 null mutant, the extra lower mobility bands were still present, which suggested that there was a delayed processing of proteases in these two mutants. Note that in each of the mutants all of these effects were reversed when the wild-type aprV5 gene was reconstituted.

AprV5 is Required for Optimal Processing of AprV2 and BprV

To further examine the effect of the C-terminal extension of AprV5 on each of the three individual proteases, equal amounts of the concentrated supernatants from the aprV5ΔC mutants were analysed on 4–15% gradient SDS-PAGE gels and blotted with specific antisera (Figure 5). Note that in these experiments the results were complicated by unavoidable immunological cross-reaction between these closely related proteases. Accordingly, the individual aprV3, aprV2 and bprV null mutants isolated previously [6] were used as negative controls alongside the wild-type positive control.

Western immunoblotting with AprV5-specific antisera showed that the mature AprV5 band produced by the aprV5ΔC1 mutant was slightly smaller in size than the equivalent band in the wild type. This band was missing in the aprV5 null mutant. By contrast, the AprV5 band in the aprV5ΔC2 and aprV5ΔC3 mutants was the same size (approx. 40 kDa) as that in the wild-type strain (Figure 5a). These results indicated that the AprV5 C-terminal extension cleavage site was located between residues 478–502, not at residue 478 as previously suggested [25]. In an attempt to determine the C-terminal cleavage site of AprV5, we introduced codons encoding a 6xHis tag onto the 3’-end of aprV5 in D. nodosus by allelic exchange, with the objective of carrying out N-terminal sequence analysis on the cleaved C-terminal extension-6xHis fragment. The resultant aprV5ΔC3 mutant (JIR3953) was confirmed as before and protease analysis on skim-milk agar showed that introduction of the 6xHis tag did not affect overall protease production (data not shown). However, Western immunoblotting using 6xHis antibody could not detect a 6xHis-tagged protein from either culture supernatants or whole cell extracts after repeated attempts, suggesting that the C-terminal extension of AprV5 was degraded after protease processing.

The results also showed that in the aprV5ΔC2 and aprV5ΔC3 strains there was a suble reduction of the amount of mature AprV5 at 16 h and 25 h, compared to the wild-type strain and the complemented derivatives. In addition, there were protein bands of higher molecular size (approx. 50 kDa) that specifically reacted with the AprV5 antiserum. We postulated that these bands represented pro-AprV5, which was supported by the observation that in aprV5ΔC1, these proteins were smaller in size than in aprV5ΔC2 and aprV5ΔC3, which was consistent with the fact that the mature AprV5 band in this strain was smaller in size. Note that all of the complemented derivatives showed a similar profile to the wild-type strain and that at 40 h all of the deletion mutants had a wild-type profile, with no larger immunoreactive bands. These data support the hypothesis that the C-terminal extension of AprV5 is required for efficient protease processing. Loss of the C-terminal extension delays processing to the mature form.

Western immunoblotting using AprV2-specific antisera confirmed that AprV3 had a role in the maturation of AprV2. The results showed that the amount of the mature AprV2 band, which migrated at approx. 38 kDa, was greatly reduced in the 16 h and 25 h cultures of the aprV5, aprV5ΔC1, aprV5ΔC2, and aprV5ΔC3 mutants and that two groups of larger proteins (approx. 65 kDa and 50 kDa) were present in these samples (Figure 5b), these were presumably less processed forms of AprV2. Similar results were obtained when BprV-specific antiserum was used, with less

---

**Figure 3. Quantitative analysis of protease activity of deletion mutants.** Total protease activity in culture supernatants was determined with azocasein as the substrate. The culture supernatants from 16 h, 25 h and 40 h TAS broth cultures of the wild type strain VCS1703A (WT), the aprV5 mutant JIR3756 (aprV5), the aprV5Δ478–595 strain JIR3947 (V5ΔC1), the aprV5Δ503–595 strain JIR3956 (V5ΔC2), the aprV5Δ60–595 strain JIR3969 (V5ΔC3) were analysed as well as their complemented derivatives: JIR3968 (V5ΔC1(V5*)), JIR3965 (V5ΔC2(V5*)) and JIR3978 (V5ΔC3(V5*)). All values were obtained from three independent biological samples. Error bars represent SEM. doi:10.1371/journal.pone.0047952.g003
processed forms of BprV also detected (Figure 5c). Once more, reconstitution of the intact aprV5 gene reversed this effect and no effects on protease processing were observed at 40 h. These data provided clear evidence that AprV5 was involved in the processing of both AprV2 and BprV. In the aprV2 and bprV mutants there was no reduction in the mature forms of the other two proteases or any evidence for the accumulation of larger proteins (Figure 5a, b & c).

Therefore, it appeared highly unlikely that AprV2 and BprV processed each other or AprV5. Western immunoblotting also was carried out on whole cell lysates of each strain using AprV2 and BprV antisera. The results showed that there were no AprV2 or BprV bands present in the whole cell lysates (data not shown), confirming that AprV2 and BprV are processed after secretion.

To confirm that the novel proteins accumulated in the aprV5 mutants were unprocessed forms of AprV2 and BprV MALDI-TOF-TOF (MS-MS) analysis was performed on in-gel proteins excised from the aprV5, aprV5D1, and aprV5D3 lanes. The results (Table 2) revealed that in the extracted 65 kDa bands both pro-AprV2-CTE and pro-BprV-CTE were identified, whereas the 50 kDa bands contained AprV2-CTE and BprV-CTE. Unfortunately, no unprocessed AprV5-derived peptides were detected, despite repeated analyses.

Discussion

Virulent isolates of D. nodosus produce three homologous extracellular subtilases, AprV5, AprV2 and BprV, each of which is synthesized as a precursor molecule that is subsequently processed to form the mature active enzyme by an unknown mechanism. In this study, we have shown that AprV5 is responsible for its own maturation and for optimal processing of AprV2 and BprV. Our results have also revealed that the C-terminal extension of AprV5 is required for efficient processing of all three enzymes, presumably because it is required for the optimal processing of AprV5. In the absence of this domain,
Table 2. Identification of unprocessed proteases in the aprV5 mutant and aprV5ΔC strains by mass spectrometry.

| Excised Gel band Size (kDa) | Protein Identification | Accession No matched Strain | Coverage % | Best Mascot Score | Peptides matched by protein region/number of confirmed sequences |
|-----------------------------|------------------------|-----------------------------|------------|-------------------|---------------------------------------------------------------|
|                             |                        |                             |            |                   | Pro-Peptides MS/MSMS  | Mature Peptides MS/MSMS  | CTE Peptides MS/MSMS |
|                             |                        |                             |            |                   |                      |                          |                  |
| 50                          | AprV2-CTE              | ASEX13                      | aprV5      | 49%               | 2/0                  | 10/6                     | 4/3               |
| 50                          | BprV-CTE               | ASEVD0                      | aprV5      | 34%               | 359                  | 4/0                      | 6/5               | 4/2             |
| 50                          | AprV2-CTE              | ASEX13                      | aprV5ΔC    | 36%               | 530                  | 0/0                      | 8/7               | 5/2             |
| 50                          | BprV-CTE               | ASEVD0                      | aprV5ΔC    | 24%               | 53                   | 0/0                      | 5/1               | 3/1             |
| 50                          | AprV2-CTE              | ASEX13                      | aprV5ΔC    | 40%               | 656                  | 2/0                      | 8/6               | 3/1             |
| 65                          | Pro-BprV-CTE           | ASEVD0                      | aprV5      | 37%               | 905                  | 3/3                      | 6/5               | 5/2             |
| 65                          | Pro-AprV2-CTE          | ASEX13                      | aprV5      | 20%               | 144                  | 2/1                      | 3/0               | 3/0             |
| 65                          | Pro-BprV-CTE           | ASEVD0                      | aprV5ΔC    | 34%               | 390                  | 3/1                      | 7/6               | 3/2             |
| 65                          | Pro-BprV-CTE           | ASEVD0                      | aprV5ΔC    | 35%               | 386                  | 3/1                      | 7/6               | 4/2             |

*Coverage denotes the percentage of the full length protein sequence that has been matched to the MS data.

**Best MASCOT score obtained between analyses from different gel pieces excised from identical gels.

\(^{1}\) MS value denotes the number of peptides matched to the MS data corresponding to sequences from within a specific region of the protein. MSMS value denotes the number of these peptides matches of which the sequences have been confirmed by MSMS analysis.

doi:10.1371/journal.pone.0047932.t002

protease processing is delayed. Finally, we have provided evidence that the cleavage of the pro-domain and the C-terminal extensions of the AprV2 and BprV precursors occurs after secretion.

It has been reported that P-domain in some bacterial subtilases is required for both the folding and regulation of pH dependence of the catalytic domain [23,34,35]. In this study we have shown that AprV5 proteins lacking the C-terminal P-domain exhibited delayed production of the mature enzyme as a result of delayed pro-protein-C-terminal extension processing. This effect was not due to a secretion defect. We postulate that the C-terminal extension may function as an intramolecular chaperon that optimizes the folding of the precursor protein prior to proteolytic cleavage.

The processing of serine proteases involves autocatalytic cleavage of each of the pro- and C-terminal domains after membrane translocation [13,36]; a similar process appears to occur for AprV5. By contrast, our results showed that the optimal maturation of AprV2 and BprV occurs at any growth stage and we could not detect a His-tagged C-terminal extension fragment in the aprV5ΔC derivative. The fact that we observed pro-mature-CTE and mature-CTE forms, but not pro-mature forms, of AprV2 and BprV in the aprV5 mutants suggests that the C-terminal extensions of AprV2 and BprV are processed after pro-region cleavage, which provides further support for the hypothesis that the C-terminal extension plays an important role in the formation of the mature enzyme. It appears that AprV2 and BprV do not process each other or AprV5 since no unprocessed proteases were detected in the aprV2 or bprV mutants, although an active band with a different electrophoretic mobility was observed in the bprV mutant, suggesting that BprV may have some role in protease processing.

It has been reported that P-domain in some bacterial subtilases is required for both the folding and regulation of pH dependence of the catalytic domain [23,34,35]. In this study we have shown that AprV5 proteins lacking the C-terminal P-domain exhibited delayed production of the mature enzyme as a result of delayed pro-protein-C-terminal extension processing. This effect was not due to a secretion defect. We postulate that the C-terminal extension may function as an intramolecular chaperon that optimizes the folding of the precursor protein prior to proteolytic cleavage.

Finally, previous studies provided evidence that the cleavage sites of the C-terminal extensions of AprV5 was at amino acid residues V477 [25]. By constructing strains expressing C-terminal truncated proteases of AprV5 we observed that the actual C-terminal cleavage site of AprV5 was different to that previously reported and is slightly closer to the C-terminus of AprV5, between residues 478 and 502.

In conclusion, we have demonstrated that the C-terminal extension of AprV5, which contains a P-domain similar to that of eukaryotic subtilisin-like proteases, is required for optimal processing and active enzyme production. We have also shown that AprV5 is responsible for the maturation of the precursors of AprV2 and BprV into their mature forms. These studies have made a major contribution to our understanding of how this pathogenic bacterium, which unusually produces three closely related subtilisin-like proteases, processes these proteases into their mature active forms.
Acknowledgments

We thank Om Dhungyel and Richard Whittington (University of Sydney) for the provision of sheep antiserum.

References

1. Stewart DJ (1989) Footrot of sheep. In: Egerton JR, Yong WK, Riffkin GG, editors. Footrot and foot abscess of ruminants. Boca Raton: CRC Press. 3–45.
2. Riffkin MC, Wang LF, Kortt AA, Stewart DJ (1995) A single amino-acid change between the antigentially different extracellular serine proteases V2 and B2 from Dichelobacter nodosus. Gene 167: 279–283.
3. Lilley GG, Riffkin MC, Stewart DJ, Kortt AA (1995) Nucleotide and deduced protein sequence of the extracellular, serine basic protease gene bprV from Dichelobacter nodosus strain 305: comparison with the basic protease gene bprF from virulent strain 198. Biochem Mol Biol Internat 36: 101–111.
4. Kortt AA, Burna JE, Vaughaq JA, Stewart DJ (1994) Purification of the extracellular acidic proteases of Dichelobacter nodosus. Biochem Mol Biol Internat 34: 1157–1166.
5. Kortt AA, Stewart DJ (1994) Properties of the extracellular acidic proteases of Dichelobacter nodosus. Stability and specificity of peptide bond cleavage. Biochem Mol Biol Internat 34: 1167–1176.
6. Kennan RM, Wong W, Dhungyel OP, Han X, Wong D, et al. (2010) The subtilisin-like protease AprV2 Is required for virulence and uses a novel disulphide-tethered exosite to bind substrates. PLoS Pathog 6: e1001210.
7. Kennan RM, Dhungyel OP, Whittington RJ, Egerton JR, Rood JI (2001) The type IV fimbrial subunit gene (fimC) of Dichelobacter nodosus is essential for virulence, protease secretion, and natural competence. J Bacteriol 183: 4451–4458.
8. Han X, Kennan RM, Davies JK, Reddacliff LA, Dhungyel OP, et al. (2008) Twitching motility is essential for virulence in Dichelobacter nodosus. J Bacteriol 190: 3323–3335.
9. Billington SJ, Johnston JL, Rood JI (1996) Virulence regions and virulence factors of the ovine footrot pathogen, Dichelobacter nodosus. FEMS Microbiol Lett 145: 147–156.
10. Rood JI (2002) Genetic islands of Dichelobacter nodosus. Curr Top Microbiol Immunol 264: 47–60.
11. Palanisamy NK, Fletcher C, Tanjung L, Katz ME, Cheetham BF (2010) Deletion of the C-terminus of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of the anaerobic bacterial pathogen Dichelobacter nodosus. FEMS Microbiol Lett 302: 39–45.
12. Bloomfield GA, Whitle G, McDonagh MB, Katz ME, Cheetham BF (1997) Analysis of sequences flanking the sap regions of Dichelobacter nodosus: evidence for multiple integration events and a new genetic element. Microbiology 143: 553–562.
13. Siezen RJ, Leunissen JA (1997) Subtilases: the superfamily of subtilisin-like serine proteinases. Protein Sci 6: 501–523.
14. Kim DW, Lee YC, Matsuawa H (1997) Role of the COOH-terminal prosequence of aqsdyn I (a heat-stable serine protease) in its extracellular secretion by Thomas thermophilus. FEMS Microbiol Lett 157: 39–45.
15. Shikata S, Shimada K, Ohnishia Y, Horinouchi S, Beppu T (1993) Characterization of secretory intermediates of Seratia marcescens serine protease produced during its extracellular secretion from Escherichia coli cells. J Biochem 114: 723–731.
16. Klauser T, Pohlner J, Meyer TF (1993) The secretion pathway of IgA protease-type proteins in gram-negative bacteria. Bioessays 15: 799–805.
17. Cedzich A, Huttenerlecher, F Kuhn RM, Pfannstiel J, Gabler L, et al. (2009) The protease-associated domain and C-terminal extension are required for zymogen processing, sorting within the secretory pathway, and activity of tomato subtilase 3 (SNBS3). J Biol Chem 284: 14065–14076.
18. Yan BQ, Chen XL, Hou XY, He H, Zhou BC, et al. (2009) Molecular analysis of the gene encoding a cold-adapted halophilic subtilase from deep-sea psychrotolerant bacterium Pseudalteromonas sp. SM9931: cloning, expression, characterization and function of the C-terminal PPC domains. Extremophiles 13: 725–733.
19. Vos P, Boerriger IJ, Bust G, Haandrijman AJ, Nijhuis M, et al. (1991) Engineering of the Lactococcus lactis serine proteinase by construction of hybrid enzymes. Protein Eng 4: 479–494.
20. Kortt AA, Riffkin MC, Focaretta A, Stewart DJ (1993) Amino acid sequence of extracellular acidic protease V3 of Dichelobacter nodosus, the causative organism of ovine footrot. Biochem Mol Biol Internat 29: 989–991.
21. Kortt AA, Caldwell JB, Lilley GG, Edwards R, Vaughan J, et al. (1994) Characterization of a basic serine protease (pl ~ 9.5) secreted by virulent strains of Dichelobacter nodosus and identification of a distinct, but closely related, protease secreted by benign strains. Biochem J 299: 521–525.
22. Wong W, Wijesuwickrema LG, Kennan RM, Reeve SB, Steer DL, et al. (2011) SI pocket of a bacterially derived subtilisin-like protease underpins effective tissue destruction. J Biol Chem 286: 42180–42187.
23. Shinde U, Thomas G (2011) Insights from bacterial subtilases into the mechanisms of intracellular chaperone-mediated activation of furin. Methods Mol Biol 768: 59–106.
24. Lilley GG, Stewart DJ, Kortt AA (1992) Amino acid and DNA sequences of an extracellular basic protease of Dichelobacter nodosus show that it is a member of the subtilisin family of proteases. Eur J Biochem 210: 13–21.
25. Riffkin MC, Focaretta A, Edwards RD, Stewart DJ, Kortt AA (1993) Cloning, sequence and expression of the gene (aprV5) encoding extracellular serine acidic protease V3 from Dichelobacter nodosus. Gene 139: 257–264.
26. Han X, Kennan RM, Parker D, Davies JK, Rood JI (2007) Type IV fimbrial biogenesis is required for protease secretion and natural transformation in Dichelobacter nodosus. J Bacteriol 191: 5022–5031.
27. Sambruk J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
28. Skinner TM (1975) Determination of some in vitro growth requirements of Bacteroides nodosus. J Gen Microbiol 87: 107–119.
29. Kennan RM, Billington SJ, Rood JI (1998) Electroporation-mediated transformation of the ovine footrot pathogen Dichelobacter nodosus. FEMS Microbiol Lett 169: 383–389.
30. Menard R, Sansonnetti P, Parsec G (1993) Nonpolar mutagenesis of the iro genes defines IpaB, IpaC, and IpaD as effectors of Aggella flexneri entry into epithelial cells. J Bacteriol 175: 5899–5906.
31. Stewart DJ (1979) The role of elastase in the differentiation of Bacteroides nodosus infections in sheep and cattle. Res Vet Sci 27: 99–105.
32. Liu D, Yong WK (1995) Use of elastase test, gelatin gel test and electrophoretic zymogram to determine virulence of Dichelobacter nodosus isolated from ovine foot rot. Res Vet Sci 55: 124–129.
33. Albert MJ, Hirdas S, Steer D, Dhaunsi GS, Smith AI, et al. (2007) Identification of a Campylobacter jejuni protein that cross-reacts with cholera toxin. Infect Immun 75: 3070–3073.
34. Seidah NG, Chretien M (1997) Eukaryotic protein processing: endoproteolysis of precursor proteins. Curr Opin Biochem 8: 602–607.
35. Zhou A, Martin S, Lipkind G, LaMendola J, Steiner DF (1998) Regulatory roles of the P domain of the subtilisin-like prohormone convertases. J Biol Chem 273: 11107–11114.
36. Coutte L, Antoine R, Drobecq H, Locht C, Jacob-Dubuisson F (2001) Subtilisin-like autotransporter serves as maturation protease in a bacterial secretion pathway. EMBO J 20: 5040–5048.
37. Bergeron F, Leudec R, Day R (2000) Subtilase-like pro-protein convertases: from molecular specificity to therapeutic applications. J Mol Endocrinol 24: 1–22.
38. Turner DF, Woodridge KG, Ali-Abedeen DA (2005) Autotransportered serine protease A of Neisseria meningitidis: an immunogenic, surface-exposed outer membrane, and secreted protein. Infect Immun 70: 4447–4461.
39. Henderson IR, Navarro-Garcia F, Nataro JP (1996) The great escape: structure and function of the autotransporter proteins. Trends Microbiol 6: 370–376.

Author Contributions

Conceived and designed the experiments: XH RMK AIS JCW JIR. Performed the experiments: XH RMK DLS. Analyzed the data: XH RMK DLS AIS JCW JIR. Wrote the paper: XH DLS JIR. Contributed to the preparation of the final manuscript: XH RMK DLS AIS JCW JIR.