Deletion of the C-terminus of polynucleotide phosphorylase increases twitching motility, a virulence characteristic of the anaerobic bacterial pathogen Dichelobacter nodosus

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Abstract
The Gram-negative anaerobe Dichelobacter nodosus is the causative agent of footrot in sheep. Different strains of D. nodosus cause disease of differing severities, ranging from benign to virulent. Virulent strains have greater twitching motility and secrete proteases that are more thermostable than those secreted by benign strains. We have identified polynucleotide phosphorylase (PNPase) as a putative virulence regulator and have proposed that PNPase expression is modulated by the adjacent integration of genetic elements. In this study, we compared PNPase activity in three virulent and four benign strains of D. nodosus and found that PNPase activity is lower in virulent strains. We disrupted the pnpA gene in three benign D. nodosus strains and two virulent strains and showed that deletion of the S1 domain of PNPase reduced catalytic activity. In all but one case, deletion of the PNPase S1 domain had no effect on the thermostability of extracellular proteases. However, this deletion resulted in an increase in twitching motility in benign, but not in virulent strains. Reconstruction of the pnpA gene in two mutant benign strains reduced twitching motility to the parental level. These results support the hypothesis that PNPase is a virulence repressor in benign strains of D. nodosus.

Introduction
Footrot is a mixed bacterial infection of the hooves of sheep, goats and deer that leads to lameness. The Gram-negative anaerobic bacterium Dichelobacter nodosus is the principal causative agent (Beveridge, 1941). Different strains of D. nodosus cause disease of differing severities, ranging from benign to virulent. The extracellular proteases secreted by virulent strains are more thermostable than proteases secreted by benign strains (Depiazzi & Richards, 1979). Virulent strains also have greater twitching motility, generated by polar type IV fimbriae, than benign strains (Depiazzi & Richards, 1985), and twitching motility is essential for virulence (Kennan et al., 2001; Han et al., 2008).

Comparative analysis of DNA from virulent and benign strains has led to the identification of a series of genetic elements that integrate into the D. nodosus chromosome. These include the intA (Katz et al., 1991, 1992, 1994; Cheetham et al., 1995; Billington et al., 1996), intB (Bloomfield et al., 1997), intC (Bloomfield et al., 1997) and intD elements (Tanjung et al., 2009), each of which contains an integrase gene. A fifth integrated element, the virulence-related locus, vrl (Katz et al., 1991; Haring et al., 1995; Billington et al., 1999), lacks an integrase gene. The distribution of these integrated elements between virulent and benign strains is nonrandom. The intA element and the vrl are found in almost all virulent strains (Rood et al., 1996; Cheetham et al., 2006) and are absent from the majority of benign strains.

The intA, intB and intD elements integrate into a tRNA-ser gene immediately downstream from pnpA. The pnpA product, polynucleotide phosphorylase (PNPase), has 3′ to 5′ exoribonuclease activity and is involved in mRNA decay initiated by endoribonucleases (Deutscher & Li, 2001). In Salmonella enterica, PNPase is a global regulator of virulence (Clements et al., 2002) that negatively regulates the expression of genes from the pathogenicity islands SPI-1 and SPI-2 (Ygberg et al., 2006). By contrast, in Yersinia spp., the PNPase S1 domain is important for the optimal functioning...
of the type III secretion system and a mutant lacking the S1 domain was found to be less virulent than the wild-type strain (Rosenzweig et al., 2007).

We have proposed that these integrated elements modulate the expression of pnpA, thereby altering the activity of PnPase, which acts as a global repressor of virulence (Whittle et al., 1999). To investigate the hypothesis that pnpA encodes a virulence regulator, we created C-terminal deletions in PNPase in several benign and virulent strains, and determined the effect on extracellular protease thermostability and twitching motility, two characteristics associated with virulence in D. nodosus. This deletion increased the twitching motility of benign strains, consistent with the hypothesis that PNPase acts as a virulence repressor in these D. nodosus strains.

Materials and methods

General methods

Methods for the growth of D. nodosus, preparation of genomic DNA, cloning and analysis of DNA, Southern blotting, DNA sequencing and DNA sequence analysis, together with the source of D. nodosus strains, have been reported previously (Katz et al., 1994; Bloomfield et al., 1997; Whittle et al., 1999). Transformation of D. nodosus used the method described by Kennan et al. (1998). Tetacycline (1 μg mL⁻¹) or kanamycin (10 μg mL⁻¹) was used to select transformants.

Identification of the D. nodosus pnpA gene

The sequence of the D. nodosus pnpA gene was extracted from the D. nodosus genome sequence (GenBank accession no. CP000513) and analysed using NCBI-ORF finder. Comparison of pnpA sequences of D. nodosus, Escherichia coli and Vibrio cholerae (GenBank accession nos. ZP_00924446 and ZP_00755444) showed that the predicted D. nodosus PnpA was very similar, consisting of 693 amino acids with five conserved domains (Fig. 1).

Construction of suicide plasmids

The construction of plasmids pCF5 and pCF7 is described in Fig. 1, and the primers are shown in Table 1. These plasmids are unable to replicate in D. nodosus, but homologous recombination at the pnpA locus may insert part or all of the plasmid into the D. nodosus chromosome. A double crossover event at pnpA would interrupt the pnpA coding
region by introducing the tetM gene after nt 891 (pCF7) or nt 1718 (pCF5) as shown in Fig. 1. Similarly, single crossover events in the 5’ segment of pnpA would disrupt the pnpA coding region at the same points, but would introduce the entire plasmid into the D. nodosus chromosome.

PNPase assay

The PNPase assay was modified from that of Fontanella et al. (1999). *Dichelobacter nodosus* cells from 16 EYE plates [Eugonagar (Becton-Dickinson) containing 2 mg mL\(^{-1}\) yeast extract and 5% v/v defibrinated horse blood] were scraped into 5 mL per plate of EYE broth [Eugonbroth (Becton-Dickinson) containing 2 mg mL\(^{-1}\) yeast extract] and collected by centrifugation at 9000 g for 5 min at 4 °C. The cells were washed three times with 1 mL of 50 mM Tris-HCl, pH 7.5, and then resuspended in 500 mL of this buffer. Aliquots of 100 mL were placed in microfuge tubes, and for each 150 mg of cell pellet, 1 g of acid-washed glass beads (212–300 μm, Sigma) were added. The cells were disrupted by vigorously shaking for 5 × 1-min periods at 4 °C, with an idle interval of 1 min in between on ice. The homogenates were incubated with 6 U of bovine pancreas DNase for 10 min at 37 °C and centrifuged at 8800 g for 20 min at 4 °C. Supernatants were extensively dialysed against 50 mM Tris-HCl, pH 7.4, and aliquots were stored at −20 °C. The protein content was assayed using the Coomassie Plus assay (Pierce), using bovine serum albumin as a standard. For the PNPase assay, the total volume was 1.5 mL, which contained 50 mM Tris-HCl, pH 7.4, 0.1 M KCl, 5 mM MgCl\(_2\), 20 μg mL\(^{-1}\) poly(A), 1.5 mM phosphoenolpyruvate, 20 mM glucose, 0.5 mM NAD+, 0.6 U mL\(^{-1}\) pyruvate kinase, 2 U mL\(^{-1}\) hexokinase, 4 U mL\(^{-1}\) glucose-6-phosphate dehydrogenase and 1–10 mg of crude protein extract. The assay mixture was incubated at 37 °C for 10 min, and then 0.75 M phosphate was added, and the absorbance at 340 nm was monitored for the next 25 min. The assay was linear over the time period of 20–35 min.

**Protease thermostability assay**

*Dichelobacter nodosus* strains were grown on EYE plates for 2 days at 37 °C. Then 5 mL of EYE broth was added to the culture plates, and they were incubated for 2 more days at 37 °C. The EYE broth was then collected from the plates into 10-mL tubes, centrifuged at 1700 g for 10 min and 0.6-mL aliquots of the supernatant were transferred to 1.5-mL microfuge tubes. Tubes were heated in duplicate at 65 °C for either 10 or 20 min while control tubes were held on ice. After heating, the tubes were transferred to ice-cold water immediately and protease activity was measured using hide-powder azure as a substrate (Depiazzi & Rood, 1984) by taking 0.5 mL of the treated supernatant and adding it to tubes containing 6 mg of hide-powder azure and 0.5 mL of protease assay buffer (10 mM HEPES, 2 mM Zwittergent 3–14, 30 mM CaCl\(_2\), pH 8.5). After mixing, the tubes were incubated at 37 °C in a shaking water bath for 30 min, then transferred to ice-cold water immediately and centrifuged at 4 °C at 8800 g for 15 min. The supernatants were transferred to 1.5-mL microfuge tubes and kept on ice. The negative control contained hide-powder azure, protease assay buffer and EYE broth. The absorbance of the supernatants at 595 nm was estimated using a Cary50 spectrophotometer. Results for the heat-treated samples were expressed as percentage of the value for the untreated samples.

**Twitching motility assay**

*Dichelobacter nodosus* strains were subcultured onto TAS agar plates (1.5% tryptone, 0.5% protease peptone, 0.2% yeast extract, 0.5% Lab-Lemco powder, 0.5% L-arginine, 0.15% dl-serine, 0.2% MgSO\(_4\)·7H\(_2\)O and 1.5% agar),
incubated under anaerobic conditions for 4 days and then used to stab-inoculate fresh TAS agar plates that were incubated for a further 4 days (Kennan et al., 2001). Brilliant blue-R dye (0.25% w/v brilliant blue-R, 40% v/v methanol and 7% v/v acetic acid) was then layered over the TAS agar plates, incubated for 30 min and then treated with destaining solution (10% acetic acid, 40% methanol, 50% water) until the blue background disappeared. The diameter of the colony was measured.

Statistical analysis

Student’s unpaired t-test was used to determine whether differences between assays were significant.

Results and discussion

Construction of suicide plasmids to disrupt pnpA

Based on similarity to other PNPases, the predicted D. nodosus PNPase has two copies of the RNAse PH domain separated by an all-α-helical core PNPase domain, which are followed by an RNAse KH domain and an RNAse S1 domain (Fig. 1). Two suicide plasmids were constructed (Fig. 1) to interrupt the PNPase-coding region after codon 297 (pCF7), which would remove the last four domains, or codon 572 (pCF5), which would remove the S1 domain.

Transformation of D. nodosus strains

The D. nodosus strains A198 and C305 are widely used as reference virulent and benign strains, respectively. Some D. nodosus strains are naturally competent, but all previous attempts to transform strains A198 and C305 have failed (Kennan et al., 1998). The transformation efficiency is very low and varies between D. nodosus strains (Kennan et al., 1998). For these experiments, the virulent strains A198, UNE61 and UNE64 (VCS1703A; Kennan et al., 1998) and the benign strains C305, 819, 1493 and 2483 were used. All strains were considered to be naturally competent (Kennan et al., 1998). Double crossover events disrupting pnpA were seen for strains UNE61, UNE64, 1493 and 2483, while a single crossover event disrupted pnpA in strain 819. No tetracycline-resistant colonies were obtained from repeated transformation experiments with pCF5 using strains A198 and C305, confirming previous results that these strains are not naturally competent (Kennan et al., 1998).

PNPase activity of parent strains and deletion mutants

The phosphorylytic activity of PNPase was measured in the benign and virulent parent strains and in the pnpA mutants (Fig. 2). PNPase activity in the virulent strain A198 was significantly lower than that in the benign strain C305, consistent with the hypothesis that PNPase acts as a virulence repressor in D. nodosus. The mean PNPase activity in the three virulent strains was 25% lower than that in the four benign strains (P < 0.05). With the exception of pnpA mutant 2483D3, all of the mutants with the C-terminal deletion in PNPase had significantly reduced PNPase activity compared with the parent strain (Fig. 2). However, this deletion reduced PNPase activity by only 20–50%. This modest reduction in PNPase activity is consistent with similar results from E. coli, where inactivation of the KH and S1 RNA-binding domains also resulted in a modest reduction in PNPase activity.
reduction in PNPase activity (Stickney et al., 2005; Briani et al., 2007). By contrast, deletion of the S1 domain of PNPase in *Salmonella* abolished phosphorylytic activity (Clements et al., 2002).

**Thermostability of extracellular proteases from parent strains and pnpA mutants**

The proteases secreted by virulent *D. nodosus* strains are, in general, more thermostable than the proteases secreted by benign strains (Depiazzi & Richards, 1979). After heat treatment, the mean remaining protease activity for the four benign strains was significantly lower than that for the three virulent strains (Table 2), as expected. Deletion of the C-terminus of PNPase did not alter the thermostability of secreted proteases from the four benign strains, or from the virulent strain UNE61, suggesting that PNPase does not act as a repressor of thermostable protease production. However, the PNPase deletion resulted in a significant reduction in protease thermostability in the virulent strain UNE64. This result is discussed in the next section.

**Twitching motility of parent strains and pnpA mutants**

The twitching motility of the benign and virulent parent strains and *pnpA* mutants was measured by determining the colony diameter after growth on TAS agar plates (Fig. 3a and c). The mean colony diameter for the four benign strains, 1.21 cm, was significantly lower than the mean colony diameter for the three virulent strains, 2.66 cm, as expected, since virulent strains have been reported previously to have greater twitching motility (Depiazzi & Richards, 1985). In four out of five benign strain *pnpA* mutants, the C-terminal deletion of PNPase resulted in a significant increase in twitching motility, consistent with the hypothesis that PNPase is a virulence repressor in *D. nodosus*. There was no significant difference between the mean colony diameter of the virulent strain UNE61 and its *pnpA* mutant.

The C-terminal PNPase deletion resulted in a decrease in twitching motility in the virulent strain UNE64. This result was unexpected, and is similar to the decrease in protease thermostability resulting from the PNPase deletion in this strain. It is possible that PNPase acts as a virulence activator in this strain. Alternatively, a mutation at a second site may have occurred during transformation.

**Reconstruction of PNPase reduces twitching motility**

To confirm that the increase in twitching motility in the benign strain *pnpA* mutants was due to the C-terminal deletion of PNPase, the PNPase gene was reconstructed in two mutants of benign strain 2483. The suicide plasmid pSK8 (Fig. 1) can undergo a double crossover at the *pnpA* and orf379 loci in the tetracycline-resistant mutants from strain 2483, to reconstruct an intact *pnpA* gene, followed by intB. As a result of this event, the tetracycline resistance gene is lost, and the kanamycin resistance gene is introduced between intB and orf379 (Fig. 1c).

Transformation of the C-terminal deletion mutants 2483D1 and 2483D2 with pSK8 resulted in approximately 200 kanamycin-resistant transformants. The transformation

| Group                              | Strains | % remaining protease activity after 10 min at 65 °C | % remaining protease activity after 20 min at 65 °C |
|------------------------------------|---------|----------------------------------------------------|---------------------------------------------------|
| Benign strains and their respective| C305    | 43.4 ± 6.6                                          | 13.0 ± 6.8                                         |
| pnpA deletion mutants              | 819     | 57.4 ± 19.0                                         | 10.4 ± 4.6                                         |
|                                   | 819D1   | 39.6 ± 8.5                                          | 7.2 ± 6.4                                          |
|                                   | 1493    | 30.7 ± 7.0                                          | 8.8 ± 1.6                                          |
|                                   | 1493D1  | 41.4 ± 7.5                                          | 8.6 ± 2.0                                          |
|                                   | 2483    | 62.4 ± 13.6                                         | 38.0 ± 16.4                                        |
|                                   | 2483D1  | 54.0 ± 11.0                                         | 23.3 ± 9.5                                         |
|                                   | 2483D2  | 57.0 ± 8.3                                          | 27.7 ± 6.7                                         |
|                                   | 2483D3  | 50.3 ± 21.0                                         | 21.7 ± 8.0                                         |
| Mean of benign parent strains      |         | 48.52 ± 14.4*                                       | 17.55 ± 13.7**                                     |
| Virulent strains and their respective| A198    | 87.4 ± 10.6                                         | 80.0 ± 9.0                                         |
| pnpA deletion mutants              | UNE61   | 80.0 ± 10.7                                         | 68.2 ± 16.4                                        |
|                                   | UNE61D1 | 87.3 ± 13.4                                         | 77.5 ± 12.6                                        |
|                                   | UNE64   | 93.0 ± 16.0*                                        | 51.7 ± 9.0*                                        |
|                                   | UNE64D1 | 62.7 ± 12.0*                                        | 31.0 ± 5.0*                                        |
| Mean of virulent parent strains    |         | 86.8 ± 6.5*                                         | 66.63 ± 14.2**                                     |

*Transformant strains with deletions in *pnpA* are designated D.

†Results are expressed as a percentage of the activity of the unheated control supernatant ± SD.

Samples paired with *, ** or † are significantly different (*P* < 0.05, Student’s t-test).
frequency of the mutants with C-terminal deletions was much greater than that of the parent strain, 2483. Thus, the decrease in PNPase activity was associated with increased competence, in contrast to *Bacillus subtilis*, where disruption of *pnpA* resulted in decreased competence (Luttinger et al., 1996). Knocking out the fimbrial subunit gene *fimA* in *D. nodosus* abolished natural competence (Kennan et al., 2001), and so it is likely that the type IV fimbriae are involved in DNA uptake and that the increased competence of mutants with C-terminal PNPase deletions is associated with their increased twitching motility.

Kanamycin-resistant transformants can be obtained using plasmid pSK81 by a variety of single or double crossover events. Of the 200 kanamycin-resistant transformants obtained, only three were sensitive to tetracycline. Southern blot analysis (data not shown) was used to show that these three transformants, 2483D1R1, 2483D2R1 and 2483D2R2, had the desired arrangement at the *pnpA* locus, resulting in reconstruction of *pnpA*.

In all three cases, the twitching motility of the strains with reconstructed *pnpA* genes was significantly less than that of the strains with C-terminal PNase deletions, and was similar to that of the parent strains (Fig. 3b and c). These results strongly suggest that the observed increase in twitching motility of the tetracycline-resistant mutants was due to the C-terminal deletion of PNase.

**Conclusions**

For the seven *D. nodosus* strains tested, we have shown that PNase activity is higher in benign strains than in virulent strains. Further, deletion of the C-terminal portion of PNase in three benign strains results in a decrease in PNase activity and an increase in twitching motility, a characteristic associated with virulence. Restoring the C-terminus to PNase in two of these mutants resulted in decreased twitching motility. These results support the hypothesis that PNase acts as a virulence repressor in these benign *D. nodosus* strains. We have proposed previously (Whittle et al., 1999) that integrated genetic elements modulate PNase activity by altering the 3’ end of *pnpA* transcripts, which may affect the stability of the mRNA or its ability to be translated. However, PNase activity may also be modified by promoter strength or amino acid sequence variation.

For one virulent strain, the PnpA deletion did not affect twitching motility, which is again consistent with the proposal that PNase is a virulence repressor. For the other virulent strain tested, the PnpA deletion resulted in decreased protease thermostability and decreased twitching motility. PNase may act as a virulence activator in this strain. Alternatively, this result may be due to a second mutation. Further investigation is needed to resolve the role of PNase in this strain.

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