The EngCP endo-\(\alpha\)-N-acetylgalactosaminidase is a virulence factor involved in \textit{Clostridium perfringens} gas gangrene infections

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\section*{ABSTRACT}

\textit{Clostridium perfringens} is the causative agent of human clostridial myonecrosis; the major toxins involved in this disease are \(\alpha\)-toxin and perfringolysin O. The RevSR two-component regulatory system has been shown to be involved in regulating virulence in a mouse myonecrosis model. Previous microarray and RNAseq analysis of a revR mutant implied that factors other than the major toxins may play a role in virulence. The RNAseq data showed that the expression of the gene encoding the EngCP endo-\(\alpha\)-N-acetylgalactosaminidase (CPE0693) was significantly down-regulated in a revR mutant. Enzymes from this family have been identified in several Gram-positive pathogens and have been postulated to contribute to their virulence. In this study, we constructed an \textit{EngCP} mutant of \textit{C. perfringens} and showed that it was significantly less virulent than its wild-type parent strain. Virulence was restored by complementation \textit{in trans} with the wild-type \textit{engCP} gene. We also demonstrated that purified EngCP was able to hydrolyse \(\alpha\)-dystroglycan derived from C2C12 mouse myotubes. However, EngCP had little effect on membrane permeability in mice, suggesting that EngCP may play a role other than the disruption of the structural integrity of myofibres. Glycerol array analysis indicated that EngCP could recognise structures containing the monosaccharide \(N\)-acetylgalactosamine at 4\(\epsilon\), but could recognise structures terminating in galactose, glucose and \(N\)-acetylglucosamine under conditions where EngCP was enzymatically active. In conclusion, we have obtained evidence that EngCP is required for virulence in \textit{C. perfringens} and, although classical exotoxins are important for disease, we have now shown that an \(O\)-glycosidase also plays an important role in the disease process.

\section*{1. Introduction}

Clostridial myonecrosis, or gas gangrene, is a histotoxic infection that is primarily caused by the Gram-positive, endospore-forming, anaerobic bacterium, \textit{Clostridium perfringens} (Rood and Cole, 1991). This rapidly progressing disease is characterised by extensive myonecrosis and gas production (Uzal et al., 2015), and is considered to be predominantly toxin-mediated (Nagahama et al., 2018; Uzal et al., 2014). \textit{C. perfringens} has the ability to produce at least 20 different toxins (Reivitt-Mills et al., 2015), of which \(\alpha\)-toxin and perfringolysin O have been implicated in clostridial myonecrosis (Awad et al., 1995, 2001; Ellemor et al., 1999; Stevens et al., 1997). The production of these toxins, as well as other potential virulence factors, involves the VnsR two-component signal transduction system (Cheung et al., 2013; Lyriris et al., 1994; Ma et al., 2011; Shimizu et al., 1994).

In addition to VnsR, the RevSR system also has been found to regulate \textit{C. perfringens} virulence in a myonecrosis model (Cheung et al., 2016; Hiscox et al., 2011). This system is comprised of the RevS sensor histidine kinase (Cheung et al., 2016) and the RevR response regulator (Hiscox et al., 2011). RNAseq analysis of a revR mutant revealed the differential expression of many genes, one of which was CPE0693 (Low et al., 2016). Previous work showed that this gene encoded an endo-\(\alpha\)-N-acetyl-D-glactosaminidase, which was called EngCP (Ashida et al., 2008). Therefore, the CPE0693 gene is hereby designated as engCP. Initial bioinformatic analysis of the amino acid sequence of EngCP indicated that it belonged to the glycoside hydrolase family 101 (GH101) (Ashida et al., 2008). Members of the GH101 family can catalyse the hydrolysis of the \(O\)-glycosidic \(\alpha\)-linkage between the galactal-\(\beta\)-1,3-N-acetyl-D-galactosamine (Gal\(\beta\)-1,3GalNAc) and the serine or threonine residue of \(O\)-linked glycans (Naumoff, 2010). This type of glycan is found predominantly in mucins and is therefore commonly known as a ‘mucin-type’ glycan (Hansisch, 2001). These \(O\)-linked glycans can be

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divided into eight different types of core structures, core 1 to core 8 (Hanisch, 2001). Purified EngCP has been shown to completely hydrolyze synthetic core 1 and core 2 substrates, and to a lesser extent, core 3 and core 8 substrates, demonstrating that EngCP has an expansive substrate specificity (Ashida et al., 2008).

To date, endo α-N-acetylgalactosaminidases containing the GH101 domain have only been identified in bacteria, both pathogenic and non-pathogenic (Garbe and Collin, 2012; Naumoff, 2010). Of those found in pathogens, an endo α-N-acetylgalactosaminidase produced by Streptococcus pneumoniae has been demonstrated to be important for pathogenesis (Marion et al., 2009). Therefore, we have examined the role of EngCP in the virulence of the C. perfringens gas gangrene isolate, strain 13. In this study, we show that EngCP is required for full virulence of C. perfringens in our mouse myonecrosis model. To investigate the potential role of EngCP in virulence, we subsequently examined the ability of purified EngCP to deglycosylate a heavily O-glycosylated protein in the sarcolemma of skeletal muscle tissue, α-dystroglycan (α-DG). This protein is part of the dystrophin-glycoprotein complex (DGC) that is essential for maintaining skeletal muscle fibre integrity (Carmignac, 2012 #1478). In the DGC of skeletal muscle, the central mucin-rich domain of α-DG interacts with laminin (Gomez Toledo et al., 2012; Ibraghimov-Beskrovnaya et al., 1992; Yoshiida-Moriguchi and Campbell, 2015) to form a crucial link between the extracellular matrix (ECM) and the intracellular cytoskeleton (Endo, 2015). We have shown that purified EngCP is able to cleave the O-glycans of α-DG, however, the O-glycan cleavage did not affect the interaction between α-DG and laminin.

2. Materials & methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this work are shown in Supplementary Table S1. E. coli strains were grown at 37 °C in SOC broth or 2x YT agar or broth (Sambrook et al., 1989) supplemented with 100 μg/ml ampicillin, 20 μg/ml kanamycin, or 30 μg/ml chloramphenicol. C. perfringens strains were cultured at 37 °C in brain heart infusion broth (BIF), fluid thioglycollate medium (FTG) (BIF), trypsin-peptone-glucose (TPG) broth (Rood et al., 1978), or nutrient agar supplemented with 30 μg/ml chloramphenicol, 10 μg/ml rifampicin and 10 μg/ml nalidixic acid, or 50 μg/ml erythromycin. All agar cultures of C. perfringens were incubated in an atmosphere of 10 % (v/v) H2 and 10 % (v/v) CO2 in N2.

2.2. Molecular techniques

The QIAprep miniprep kit (Qiagen) was routinely used to isolate plasmid DNA from E. coli cells as per the manufacturer’s instructions. Restriction endonucleases (Roche, New England Biolabs), DNA ligase (Promega), and Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific) were used as specified by the manufacturers. PCR amplified products were extracted from 0.8 % (w/v) agarose gels using the UltraClean® 15 DNA purification kit (Mo Bio Laboratories) as per the manufacturer’s instructions. All PCR amplified products were sequenced to ensure that no errors had been incorporated. The oligonucleotide primers used in this study are listed in Supplementary Table S2.

DNA sequencing reactions were performed using the PRISM BigDye Terminator Mix (Applied Biosystems), and signal detection was carried out by Micronem (Monash University) on an Applied Biosystems 3730 Genetic Analyzer. Nucleotide sequences were analyzed using Sequencher™ software (Gene Codes) or ContigExpress (Invitrogen).

Chemically competent E. coli cells (Inoue et al., 1990) and electro-competent C. perfringens cells (Scott and Rood, 1989) were made and transformed as described. C. perfringens genomic DNA was isolated from 5 ml of an FTG culture as described previously (O’Connor et al., 2006).

2.3. Construction of the engCP Targetron plasmid

The group II intron was re-targeted to insert between positions 876/877 (relative to the ATG start codon) in the sense DNA strand of engCP. To obtain the re-targeted 349 bp fragment, splice overlap extension PCR was carried out using Phusion High Fidelity DNA polymerase and the Targetron mutagenesis primers in Supplementary Table S2. The resultant PCR product was cloned into the HindIII/BsrGI sites of pJJR3566 as described previously (Cheung et al., 2010) to give pJJR4466, which was then used in the construction of the engCP mutant of the wild-type strain, JIR325.

2.4. Construction and complementation of the engCP mutant

The engCP mutant, JIR13229, was constructed by Targetron insertion using pJJR4466, as before (Cheung et al., 2010). The engCP mutant was confirmed by PCR analysis and Southern hybridization (data not shown). To construct the engCP complementation plasmid, the engCP gene, with its promoter region, was PCR amplified using primers JRP6441 and JRP6442, which introduced Asp718 and BamHI sites, respectively. The resultant 5.4 kb product was digested and ligated into the corresponding sites of pJJR750, to give pJJR4650. This engCP complementation plasmid, as well as pJJR750, were used to transform JIR13229 to produce the engCP complementation strain (JIR13441) and the vector control strain (JIR13439), respectively.

2.5. Construction of engCP and nanl expression plasmids

To facilitate cloning of the 4.98 kb engCP fragment into pET-22b(+) (Novagen), the primers JRP6766 and JRP6707 (Supplementary Table S2) incorporated an NdeI and a XhoI restriction site at the 5’ and 3’ end of the PCR product, respectively. Similarly, to enable the in-frame insertion of the 2.0 kb nanl fragment into pET-28b(+) (Novagen), the primers, JRP6781 and JRP6782, introduced an Nost and a XhoI restriction site at the 5’ and 3’ end, respectively. Note that both genes were amplified without their predicted signal sequences at the 5’ end of the gene. The resultant engCP (pJJR4749) and nanl (pJJR4758) expression constructs were sequenced to ensure that the genes were free of mutations and in the correct reading frame.

2.6. Overexpression and purification of His-tagged EngCP and Nanl

To overexpress the His-tagged EngCP (~186 kDa) and Nanl (~76 kDa) proteins, E. coli BL21(DE3) C43 cells were transformed with pJJR4749 or pJJR4758, respectively. The resultant strains were cultured overnight at 30 °C in autoinduction media, which consisted of 2x YT broth supplemented with the appropriate antibiotics, 1 mM MgSO4, and the P and 5052 solutions (Studier, 2005). Following induction, cells were harvested by centrifugation at 15, 300 g for 15 min at room temperature.

To purify EngCP and Nanl, the harvested cells were resuspended in lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 10 % (v/v) glycerol, cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche), pH 7.5 containing 0.01 % (v/v) Triton X-100, and 10 mM imidazole (EngCP) or 5 mM imidazole (Nanl)). Cells were lysed using an Emulsiflex C5 high pressure homogenizer (Avestin), followed by clearing by centrifugation at 12, 000 g for 20 min at 4 °C. Proteins were purified using Talon® Superflow metal affinity resin (Clontech).

For the purification of EngCP, the resin was washed with wash buffer (lysis buffer supplemented with 20 mM imidazole), and the protein was eluted with elution buffer (lysis buffer supplemented with 250 mM imidazole). EngCP was further purified by passing a pooled and concentrated preparation through a Superdex S200 gel filtration column (GE Life Sciences) and eluted with storage buffer (20 mM Tris-
HCl, 200 mM NaCl, 10 % (v/v) glycerol, pH 7.5).

His-tagged Nanl was purified using the same method, except that the buffers did not contain the protease inhibitor cocktail, and the resin was washed with wash buffer supplemented with 5 mM imidazole. After eluting with elution buffer containing 250 mM imidazole, the pooled and concentrated Nanl preparation was further purified by the Protein Production Unit (Monash University) using a Superdex S200 gel filtration column. Fractions containing Nanl eluted in storage buffer were pooled and concentrated using an Amicon Ultra-15 concentrator (Millipore), as per the manufacturer’s instructions. Purified protein concentrations were determined with the Pierce BCA protein assay kit (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions.

2.7. Bioinformatic analysis

Analysis of the depth of coverage plots of RNAseq data generated previously (Low et al., 2016) was carried out in the Artemis genome browser (Carver et al., 2012). Domain searches were carried out using the InterPro algorithm (Mitchell et al., 2015, 2018). SignalP v 4.1 (Petersen et al., 2011) and PSORT v 3.0 (Yu et al., 2010) algorithms, and the TOPCONS server (Tsirigos et al., 2015) were used to predict the topology and localization of the mature EngCP and Nanl proteins. Inverted repeats in the engCP promoter region were identified using the EMBOSS palindrom prediction algorithm (Rice et al., 2000).

2.8. Virulence testing of isogenic C. perfringens strains

To test the effect of the engCP mutation on virulence, isogenic C. perfringens strains were assayed in a murine myonecrosis model using 6–8 week old female BALB/c mice. Approximately 10^6 colony forming units (CFU) of each C. perfringens strain was injected into the right hind muscle, and mice were subsequently monitored and scored hourly using disease parameters described previously (Cheung et al., 2016; Hiscox et al., 2011). Mice were euthanized humanely upon severe disease onset, in accordance with Victorian State Government regulations and with the approval of the Monash University MARP-2 Animal Ethics Committee (Ethics number MARP/2016/146). Following euthanasia, infected thigh muscle tissues were dissected and used to determine the bacterial load using viable counts (Hiscox et al., 2011). The log-ranked Mantel Cox test and Mann Whitney U test in the GraphPad Prism 6 software were used to determine the statistical significance of the Kaplan-Meier survival curves and survival time graphs, respectively.

2.9. Analysis of membrane permeability using Evans Blue dye in the mouse myonecrosis model

To test the permeability of the membrane surrounding muscle fibres, 6–8 week old female BALB/c mice were injected intraperitoneally with 50 μL of Evans Blue dye (EBD) solution (1 % (w/v) EBD in PBS; sterilized by passage through a 0.22 μm filter) per 10 g body weight ~16 h before the mice were injected intramuscularly with the C. perfringens strains. Mice were scored and monitored hourly for disease progression and were subsequently humanely killed at 4 h post injection. This time point was chosen since previous experiments with mice injected with the wild-type C. perfringens strain showed that the average survival time was approximately 4.5 h. Both the infected and uninfected right hind thigh muscles were dissected for EBD quantification as described below.

To quantify the amount of EBD in muscle tissues, the dissected tissues were frozen at ~80 °C for 24 h before being freeze dried using an Econo freeze dryer (Thermoline Scientific) for 24 h. Freeze-dried muscle tissues were then cut into smaller pieces, weighed and macerated in 1 ml of 100 % formamide (Sigma). To extract the EBD, samples were incubated at 60 °C for 2 h, followed by an additional overnight incubation at room temperature with continuous rotational mixing. Samples were centrifuged at 10,000 g for 40 min at room temperature, and 100 μl of each supernatant was transferred to a 96 well microtitre plate (Falcon) in duplicate. Absorbance of the supernatants was measured against a formamide blank at 620 nm in a Tecan Infinite M200 microtiter plate reader. Concentrations of EBD were determined by comparison to a standard curve of 0.2 μg/ml – 12.5 μg/ml of EBD in formamide. The amount of EBD in the infected tissues was shown as micrograms of EBD per gram of dry muscle.

2.10. Glycan array analysis

Glycan arrays were printed using an ArrayIt Spotbot Xtreme array printer and processed as previously described (Waespy et al., 2015). The process is also outlined in the MIRAGE compliance table provided in Supplementary Table S3. Array analysis was conducted as previously described (Shewell et al., 2014) with the following modifications. Glycan arrays were blocked in cold 0.5 % (w/v) BSA in array PBS (1x PBS with 1 mM CaCl_2 and 1 mM MgCl_2) for 5 min. EngCP (2 μg) was mixed with a rabbit anti-His antibody (Cell Signalling), a goat anti-rabbit AF647 antibody (Thermo Fisher), and a donkey anti-goat AF647 antibody (Thermo Fisher) and allowed to complex for 5 min in array PBS on ice or at room temperature in the dark. The protein-antibody complex was then incubated on the glycan arrays for 15 min on ice and on the glycan arrays for 15 min at room temperature. The arrays were then washed for 5 min in order, in cold 0.1 % BSA in array PBS, 0.001 % (v/v) Tween_20 in array PBS, and array PBS. The arrays were then centrifuged at 100 g for 3 min to dry. Glycan arrays were scanned using an Innoscan 1100AL three laser scanner and processed using Innopsys Mapix data acquisition and analysis software and Microsoft Excel for statistical analysis.

2.11. Culturing and differentiation of C2C12 cells

C2C12 mouse muscle myoblast cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma), and 10 % (v/v) heat inactivated foetal calf serum (Gibco/Invitrogen). To induce differentiation of cells to myotubes, myoblasts were cultured to ~90 % confluence before changing to differentiation medium consisting of DMEM supplemented with 2 % heat inactivated horse serum (Gibco/Invitrogen). Cells were differentiated for 5 days, with the media changed every 48 h. All cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2.

2.12. Isolation of C2C12 myotube lysates

C2C12 myoblast cells were seeded in 6 well tissue plates (Falcon) at a density of 2.5 x 10^5 cells/ml. After 5 days of differentiation, myotubes were washed with ice cold PBS, incubated with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % (v/v) Triton X-100, 1mM EDTA-free protease inhibitor cocktail) for 10 min on ice, before being scraped off and transferred to a 1.5 ml microcentrifuge tube. Complete cell lysis was achieved by incubating samples at 4 °C for 1 h with continuous rotation. After centrifugation at 16,000 g for 15 min at 4 °C, the solubilised proteins were recovered in the supernatant. Total protein concentration was determined using the BCA protein assay kit.

2.13. PNA lectin blot, laminin overlay, and Western blot analysis

To obtain de-O-glycosylated proteins for all blotting assays, 50 μg samples of the myotube lysates were either untreated, treated with 0.5 μM Nanl or 0.27 μM EngCP, or both 0.5 μM Nanl and 0.27 μM EngCP using the buffers and protocol of the O-glycosidase and neuraminidase bundle from New England Biolabs. Following incubation at 37 °C for 4 h, de-O-glycosylation samples were resolved on 10 % SDS-PAGE gels
alongside 0.5 μM NaN₃, 0.27 μM EngCP or 1.8 μg of natural mouse laminin (Invitrogen). The proteins were subsequently transferred to nitrocellulose membranes in Towbin transfer buffer (Towbin et al., 1979) at 4 °C and used in the following blotting assays.

PNA lectin blotting was carried out by blocking the nitrocellulose membrane with PBS containing 0.05% (v/v) Tween₂₀ (PBS-T), followed by incubation with 1 μg/ml of peroxide-conjugated peanut agglutinin (PNA) lectin (Sigma) in PBS-T. Both steps were carried out at room temperature for 1 h. Detection was performed using the Western lightning Plus ECL reagent (Perkin Elmer) in accordance with the manufacturer's instructions.

The laminin overlay assay was performed by blocking the nitrocellulose membrane with laminin binding buffer (LBB) (20 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, pH7.4) containing 5% (w/v) skim milk for 1 h at room temperature. After washing briefly with LBB, the membrane was incubated with LBB containing 3% (w/v) BSA and 1.2 μg/ml natural mouse laminin overnight at 4 °C. To remove unbound laminin, the membrane was washed (3 × 10 min) with LBB at room temperature. Laminin binding was detected by incubating the membrane with LBB containing 0.05% (v/v) Tween₂₀ (LBB-T), 5% (w/v) skim milk and 1 μg/ml anti-laminin 1 + 2 antibody (Abcam), followed by incubation with LBB-T containing 5% (w/v) skim milk and 1:5000 diluted horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Millipore). Both steps were carried out at 37°C for 2 h, with three 10 min washes at room temperature with LBB-T before and after the secondary antibody step. The blot was developed with the Western lightning Plus ECL reagent.

Western blot analysis was carried out alongside the PNA lectin blot and laminin overlay analysis. The nitrocellulose membrane was blocked for 1 h at room temperature with TBS (50 mM Tris-HCl, 150 mM NaCl, pH7.4) containing 0.05% (v/v) Tween₂₀ (TBS-T) and 5% (w/v) skim milk, followed by overnight incubation at 4°C with a 1:1000 dilution of anti-α-dystroglycan IHHSC4 antibody (Millipore) in blocking buffer. The membrane was then incubated for 1 h at room temperature with TBS-T containing 1:3000 diluted horseradish peroxidase-conjugated anti-mouse secondary antibody (Millipore), before being developed with the Western lightning Plus ECL reagent.

3. Results

3.1. EngCP contains several potentially functional domains

Analysis of the depth of coverage plots of the wild type, revR mutant and virR mutant, generated from previous RNAseq data (Low et al., 2016), showed that the 5.061 kb engCP gene was not co-transcribed with any upstream or downstream genes (Fig. 1A). Comparative examination of these plots showed that engCP was expressed in the wild type and virR mutant, but not in the revR mutant, indicating that its transcription was RevR-regulated (Fig. 1A). We have previously shown that RevR recognises and binds to PHO box-like repeats that are located near the -10 and -35 box promoter of genes that are directly regulated by RevR (Cheung et al., 2016). Although the engCP promoter sequence had repeats that were similar to the RevR binding site, their spacing, arrangement and location was not consistent with those found previously (Cheung et al., 2016). This finding suggested that engCP was indirectly regulated by RevR. In addition to these repeats, three sets of inverted repeats (IR1, IR2 & IR3) were identified in this promoter region (Fig. 1B). Whether these repeats are involved in the transcriptional regulation of engCP remains to be determined.

The predicted molecular mass of the full length EngCP protein was ~187.8 kDa. Bioinformatic analysis using the SignalP, TOPCONS, and PSORTb algorithms indicated that EngCP contained an N-terminal signal peptide (Fig. 1C), did not harbour any transmembrane domains, and had an extracellular location, respectively. Secreted EngCP was predicted to be ~186 kDa in size. The signal sequence, as well as an Ig-like domain, a GH101 conserved region containing a carbohydrate binding module (CBM 4.9), and a fibronectin type III (FN3) domain, was identified previously (Ashida et al., 2008). Our bioinformatic analysis of EngCP (Fig. 1C) revealed that the GH101 conserved region was comprised of a glycoside hydrolase-type carbohydrate binding superfamily (GHB) domain, an endo α-N-acetylglucosaminidase domain (ENG), an FN3 domain, and two tandemly arranged galactose binding domains (GBD), the first of which was the CBM 4.9 domain identified previously (Ashida et al., 2008). The second GBD belonged to the galactose binding-like domain superfamily. Note that the amino acid sequences of some of these domains were predicted to overlap (Fig. 1C).

In addition, we also identified a Concanavalin A-like lectin/glucanase domain superfamily (ConA) domain in the N-terminal region. These data suggested that EngCP was likely to be a complex multifunctional carbohydrate-binding protein.

3.2. Mutation of engCP reduces the virulence of C. perfringens

To test whether EngCP was involved in the virulence of C. perfringens, an engCP mutant was constructed by Targetron insertion into the sense strand between nucleotides 876 and 877, with respect to the start of the engCP gene. The resultant engCP mutant was validated using PCR and Southern blot analysis (data not shown) and subsequently complemented in trans with a plasmid carrying a wild-type engCP gene. In vitro growth curve analysis showed that the engCP mutant and complemented strain grew at a similar rate to the wild type, while qualitative analysis showed that perfringolysin O and α-toxin levels were similar in all strains (Supplementary Fig. S1A & S8). These results showed that mutation of engCP did not affect these characteristics.

The isogenic wild-type, engCP mutant and engCP complemented strains were tested in our mouse myonecrosis model, with the disease parameters scored hourly for 12 h (Fig. 2A). The results showed that for all animals, mice injected with the wild-type and engCP complemented strains showed the greatest level of disease, while the mice injected with the engCP mutant showed slower disease progression and a lower level of disease. In particular, mice injected with the engCP mutant showed noticeably less swelling and blackening of the footpad (Fig. 2A); the latter is used as an indicator of necrotic disease-induced ischaemia. The uninfected control mice, which were injected with PBS, did not show any disease symptoms.

Mice injected with the wild-type strain started to succumb to disease at approximately 3 h post-injection, with all mice having to be euthanized before the end of the 12 h trial period for ethical reasons (Fig. 2B). By comparison, most of the mice injected with the engCP mutant survived to the end of the trial (Fig. 2A). When the engCP mutation was complemented, virulence was restored, as all mice injected with the complemented strain succumbed to disease before the end of the trial (Fig. 2B). Log-ranked Mantel-Cox analysis of the Kaplan-Meier survival curves showed that the engCP mutant was significantly attenuated for virulence compared to the wild-type (p < 0.0001) and engCP complemented (p < 0.0001) strains. By contrast, there was no significant difference between the virulence of the wild type compared to the complemented mutant (p = 0.76). Mice injected with the wild type and complemented mutant had similar (p = 0.36, Mann-Whitney U test) mean survival times of 4.8 ± 0.3 h and 4.5 ± 0.4 h, respectively (Fig. 2C). Conversely, the mean survival time of mice injected with the engCP mutant was 10.4 ± 0.9 h, which was significantly different to the mean survival times of the mice injected with the wild type (p < 0.0001) or engCP mutant (p < 0.0001). Quantification of the bacterial load from infected muscle tissue showed no statistical difference in viable counts (p > 0.05, One way ANOVA with multiple comparisons) (Supplementary Fig. S1C), suggesting that the difference in virulence was not due to reduced cell numbers. Overall, these results demonstrated that mutation of engCP leads to an attenuation in virulence, which could be restored to wild-type levels by complementation with the wild-type gene.
3.3. EngCP hydrolyses the O-GalNAc core structures of α-dystroglycan (α-DG)

In skeletal muscle tissue, the site of gas gangrene infections, α-DG is one of the most heavily O-glycosylated proteins. This protein is found in the sarcolemma, which is the membrane surrounding each skeletal muscle fibre (Endo, 2015). Previous in vitro cell culture work showed that glycosylated α-DG was predominantly found in differentiated C2C12 myotubes compared to undifferentiated myoblasts (Kostrominova and Tanzer, 1995). Given that previous work had shown that EngCP could cleave a broad range of O-glycans (Ashida et al., 2008) we tested whether EngCP had an effect on the O-glycosylation state of α-DG. Purified EngCP, in combination with purified Nnal siaidase, was used to treat lysates derived from 5 day old C2C12 myotubes. Nnal was included since synergism between EngCP and siaidase has been observed previously (Ashida et al., 2008). Because the hydrolysis and release of small O-linked glycans would not result in a significant shift in electrophoretic mobility on SDS-PAGE, lectin blotting using peroxide-conjugated peanut agglutinin (PNA), which binds to unmodified Gal-β1,3GalNAc disaccharides, was used to visualise the release of Gal-β1,3GalNAc disaccharides from solubilised myotube proteins (Fig. 3A). To show the position of the α-DG protein, Western blotting using the IIH6 α-DG antibody was carried out in parallel on duplicate samples (Fig. 3B).

A band of the size expected for α-DG was not observed in the PNA lectin blot of the untreated myotube sample (Fig. 3A), as anticipated, since PNA cannot bind to Gal-β1,3GalNAc disaccharides modified with a terminal sialic acid (McDearmont et al., 2003). When the myotube lysate was treated with Nnal, a band with an electrophoretic mobility that was consistent with desialylated α-DG was observed when probed with PNA (Fig. 3A). This result indicated that the purified Nnal protein could cleave the terminal sialic acids to reveal Gal-β1,3GalNAc disaccharides to which PNA could bind. The desialylation of α-DG was reflected in the altered electrophoretic mobility of the α-DG band on the parallel Western blot. When the myotube lysate was treated with EngCP, the α-DG band was not observed in the PNA lectin blot. This result implied that EngCP was not able to cleave sialylated α-DG. The absence of the α-DG band was not due to the absence of protein, since α-DG was detected in the parallel IIH6 antibody Western blot (Fig. 3B). Only a very faint α-DG band was observed when the myotube lysate was treated with both Nnal and EngCP. In this sample, Nnal presumably had removed the sialic acids from α-DG, thereby exposing the Gal-β1,3GalNAc disaccharides, which in turn were liberated by EngCP. The release of these disaccharides would mean that PNA was unable to bind to α-DG. This reaction showed that EngCP was functional, since a strong α-DG band, like that observed on the corresponding Western blot, would have been observed if EngCP had not hydrolysed the core 1 glycans. Note that in each lane, PNA also bound to other proteins that had exposed Gal-β1,3GalNAc disaccharides, resulting in the additional bands. On both the lectin and Western blots, the Nnal siaidase consistently reacted non-specifically, while faint EngCP bands also were observed.
3.4. EngCP does not affect laminin binding to α-DG

Glycosylation of α-DG has been found to be essential for laminin binding to α-DG (Endo, 2015). To examine whether this binding was affected by EngCP-mediated de-O-glycosylation of α-DG, a laminin overlay blot was performed (Fig. 3C). To visualise the α-DG protein, Western blotting using the I1H6 α-DG antibody was carried out in parallel on duplicate samples (Fig. 3D).

The results showed that laminin in the overlay was able to bind to α-DG in the untreated myotube lysate (Fig. 3C). When the myotube lysate was treated with Nαl, an α-DG band of altered electrophoretic mobility was observed, indicating that laminin could bind to desialylated α-DG. When the myotube lysate was treated with EngCP alone or in conjunction with Nαl, laminin was still able to bind to α-DG. The latter reaction demonstrated that laminin was able to bind to α-DG even when the Gal-β1,3GalNAc disaccharides had been released by EngCP, indicating that EngCP does not affect the core M3 glycan of α-DG that is recognised and bound by laminin. The presence of α-DG in all lanes was confirmed by the Western blot with the I1H6 α-DG antibody.

Note that non-specific binding of the laminin antibody to purified EngCP was observed in lanes containing the latter protein, which was not due to the binding of laminin to EngCP, since the band also was observed when Western blotting with the laminin antibody was carried out (data not shown). By contrast, only a very faint purified Nαl band was observed on the overlay blot, indicating that the non-specific binding of the laminin antibody to EngCP was not due to the His-tag. As expected, control laminin proteins in the overlay blot reacted with the laminin antibody (Fig. 3C).

3.5. EngCP does not contribute to sarcolemma damage in necrotic skeletal muscle

O-glycosylation of α-DG plays an important role in the maintenance of sarcolemma integrity (Muntoni et al., 2008). To examine the effect of EngCP on myofibre membrane permeability, mice were injected intraperitoneally with Evans Blue dye (EBD). This dye has been used extensively as a tracer to assess the integrity of cell membranes in vivo, as it is excluded from cells that have an intact membrane but is able to enter and accumulate in cells with damaged membranes (Woodell et al., 2011). Approximately 16 h after the injection of EBD, the mice were injected with isogenic C. perfringens strains, then monitored and scored hourly for 4 h (Fig. 4A). During this time, the other disease parameters developed in a similar way to the corresponding time points in the full 12 h virulence trials. That is, the EBD mice that were subsequently injected with the wild-type or complemented mutant strains showed faster disease progression, which developed into more severe disease compared to the mice injected with the engCP mutant. The uninfected control mice that were injected with PBS did not show any disease at all time points.

At 4 h post injection of the bacterial strains, the mice were
Fig. 3. Effect of EngCP and Nanl on the glycosylation state of α-DG. (A) PNA lectin blot with the corresponding α-DG IIH6 Ab western blot (B). (C) Laminin overlay with the corresponding α-DG IIH6 Ab western blot (D). The presence (+) or absence (-) of 0.5 μM of Nanl, 0.27 μM of EngCP, 1.8 μg of laminin (Lam), and 50 μg of myotube lysate (MT) are shown above each blot, as is the Precision Dual Color standard (Std). The location of the EngCP, Nanl and α-DG protein bands is indicated by the arrows.

3.6. EngCP recognises and binds to glycans containing galactose, N-acetylgalactosamine and N-acetylglucosamine

Since EngCP was required for full virulence, but did not significantly contribute to muscle damage, glycan array analysis was carried out to provide insight into its potential target(s). Bioinformatic analysis of EngCP showed the presence of several carbohydrate binding domains, as well as the enzymatic ENG domain. To determine which glycans were bound and/or cleaved by EngCP, a His-tagged EngCP protein was purified by metal affinity chromatography and subsequently used in glycan array analysis at two different conditions, on ice (4 °C) and at room temperature (25 °C) (Dataset S1). The results obtained at 4 °C (limited enzyme activity) and 25 °C (more active enzyme) incubation identified the glycan structures on the array that were able to be recognised, but not enzymatically cleaved, and those that could be bound for enzymatic function, respectively (Table 1). At 4 °C, EngCP recognised 23 structures (Table 1), but binding to eight of these glycans was lost when the array analysis was carried out at 25 °C. These eight structures included five glycans containing β1-3GalNAc (Glycan IDs 89, 255, 262, 362 and 17 G; Table 1). Binding to three hyaluronic acid structures was also lost at 25 °C (13 N, 14 F and 14 J; Table 1), suggesting a potential role for EngCP in the hydrolysis of hyaluronic acid in the extracellular matrix. The structures recognised at the lower temperature, but which were not cleaved, comprised a range of core type 1 and 2 (Galβ1-3/4GlcNAc) structures, including blood group and Lewis antigens and other components of N-linked glycans including β-mannose, which do not normally contain the GalNAc motif recognised by EngCP for cleavage (Stanley et al., 2009). Structures that contained the
β1-3GalNAc motif that were not cleaved included glycan 317. This glycan is modified with both sialic acid and sulfation, providing further evidence that EngCP requires the action of neuraminidase prior to cleavage.

4. Discussion

Gas gangrene, or clostridial myonecrosis, is regarded as a toxin-mediated infection (Rood and Cole, 1991), where α-toxin and

Table 1
Glycan array analysis of EngCP at 4 °C and 25 °C.

<table>
<thead>
<tr>
<th>Array ID</th>
<th>Glycan</th>
<th>Structure</th>
<th>4 °C</th>
<th>25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>α-Galactose</td>
<td>Gala-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>β-Mannose</td>
<td>Manβ-sp4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>37</td>
<td>3-O-Sulβ-Galactose</td>
<td>3-O-Sulβ-Galβ-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>45</td>
<td>β-glucuronic acid</td>
<td>GlcAβ-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>71</td>
<td>Blood Group H Antigen</td>
<td>Galβ1-3GlcNAcβ-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>85</td>
<td>Lewis C</td>
<td>Galβ1-3GlcNAcβ-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>89</td>
<td>Thomsen-Friedenreich antigen</td>
<td>Galβ1-3GlcNAcα-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>225</td>
<td>P1 Antigen</td>
<td>Galα1-4Galβ1-4GlcNAc-sp2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>241</td>
<td>Isomaltotriose</td>
<td>(Glcα1-6)β4-sp4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>251</td>
<td>GlcNAcβ4 Lactosamine</td>
<td>GlcNAcβ1-4Galβ1-4GlcNAcβ-sp2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>252</td>
<td>Chloritrose</td>
<td>GlcNAcβ1-4GlcNAcβ1-6GlcNacβ-sp4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>250</td>
<td>core 4</td>
<td>GlcNAcβ1-3-GlcNAcβ1-6GlcNAcα-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>262</td>
<td>TpeGal</td>
<td>Galβ1-3GalNAcβ1-3Galα4-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>287</td>
<td>Sulfated Lewis A Antigen</td>
<td>3-O-Sulβ-Galβ1-3(Fucα1-4)GlcNAcβ-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>296</td>
<td>3 Sialyllactosamine</td>
<td>Neu5Aco2-Galβ1-3GlcNAcβ-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>317</td>
<td>6-Sul-3SLacet</td>
<td>Neu5Aco2-3Galβ1-3Galβ1-3GlcNAcα-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>362</td>
<td>Blood Group B Antigen, type III</td>
<td>Galα1-3(Fucα1-2)Galβ1-3GlcNAcα-sp3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13L</td>
<td>Dermatan Sulfate</td>
<td>(l ± 2S)GlcA/IdoAα2-3β1-(3 ± 4S)GlcNAcαβ1-4, (n &lt; 250)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13M</td>
<td>Chondroitin 6-Sulfate</td>
<td>(GlcA/IdoAβ1-3(± 6S)GlcNAcβ1-4, (n &lt; 250)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13 N</td>
<td>Hyaluronic acid (HA)-4</td>
<td>(Glcβ1-4GlcNAcβ1-3)yNH2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14 F</td>
<td>HA 107000 da</td>
<td>Glcβ1-4GlcNAcβ1-3n</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14 H</td>
<td>HA 169000 da</td>
<td>(Glcβ1-4GlcNAcβ1-3s)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>171</td>
<td>fucosyl GM1 ganglioside sugar</td>
<td>Fucα2Galβ1-3GlcNAcβ1-4( Neu5Aco2-3Galβ1-4Glc</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates binding across three independent array experiments.
perfringolysin O act synergistically to cause disease (Awad et al., 2001). However, analysis of a revR mutant showed that although it was able to produce both toxins, the strain was attenuated for virulence (Hiscox et al., 2011), suggesting that other virulence factors may also play an important role in disease pathogenesis. In this study, we have shown that EngCP, an endo α-N-acetylglucosaminidase belonging to the GH101 family, is required for the full virulence of C. perfringens. To date, members of the GH101 family of endo α-N-acetylglucosaminidases have been identified in over 400 bacterial isolates in the Carbohydrate-Active enZymes (CAZY) database (www.cazy.org). Of the enzymes from pathogenic bacteria, only SP0568 (EngSP) from S. pneumoniae has been demonstrated to be involved in pathogenesis (Marion et al., 2009), while the endo α-N-acetylglucosaminidase from Melissosoccus plutonus has been hypothesised to be a virulence factor (Djukic et al., 2018). In this work, when the engCP gene was insertionally inactivated, the resultant engCP mutant was shown to be significantly reduced in virulence in our mouse myonecrosis model, with a concomitant increase in the survival time compared to the mice injected with the wild-type and EngCP complemented strains. The reduction in virulence was not attributed to defects in growth or toxin production, since similar viable cell numbers were obtained from all the infected tissues examined, and the α-toxin and perfringolysin O levels were not altered significantly. This study demonstrates, for the first time, the clear involvement of a C. perfringens virulence factor in myonecrotic disease that is not classified as a toxin.

EngCP was initially annotated as a hypothetical protein (Shimizu et al., 2002). However, subsequent studies on EngCP derived from two different gas gangrene isolates indicated that it was an endo α-N-acetylglucosaminidase that had a broad substrate specificity, with a preference for core 1 O-glycans (Ashida et al., 2008; Koutsioulis et al., 2008). The results of our glycan array analysis showed that EngCP was able to bind to galactose, N-acetylgalactosamine, glucose, glucuronic acid, β-mannose and N-acetylglucosamine, which are sugars found in O-glycans (Brockhausen and Stanley, 2015), N-glycans (Stanley et al., 2009) and glycosaminoglycans (GAGs) (Lindahl et al., 2015). This result is consistent with the finding that there are several galectose carbohydrate binding domains within the predicted amino acid sequence of EngCP. Our bioinformatic analysis showed that in addition to the domains identified previously (Ashida et al., 2008), EngCP also contained a region belonging to the Concanavalin A-like lectin/glucosaminidase domain superfamily. Members of this family share a sandwich structure consisting of 12–14 β-strands in two sheets (Williams and Westhead, 2002). This structure can also be found in several different glycosyl hydrolases, lectins, the laminin G-like module of laminin, sialidases, and several bacterial toxins, including clostridial neurotoxins (Chandra et al., 2001). Although the precise function of this domain has not been elucidated, lectin-like domains have been identified in the receptor binding regions of several virulence factors (Swaminathan and Eswaramoorthy, 2000) (Moustafa et al., 2004; Wedekind et al., 2001).

In addition to the FN3 domain at the C-terminal end of EngCP, we identified another FN3 domain in between the ENG domain and first of the two galactose-binding domains. The function of FN3 domains in bacterial proteins remains unclear. Although it was initially postulated that these domains were involved in substrate binding (Little et al., 1994), it is now thought that the FN3 domains in bacterial carbohydrates may act as stable linkers that position enzymatic domains for optimal function (Valk et al., 2017). At present, the role of these domains in EngCP is unknown. However, comparison of the predicted domains of EngCP with those of EngSP (S. pneumoniae), EngEF (Enterococcus faecalis) and EngBF (Bifidobacterium longum), the activities of which have been characterised and compared to those of EngCP (Koutsioulis et al., 2008), showed that the ConA and FN3 domains were only found in EngCP (data not shown), suggesting that these two motifs may be important for specific EngCP function.

Previous work showed that purified EngCP could hydrolyse O-glycans located on pre-treated porcine gastric mucin (Ashida et al., 2008). In skeletal muscle tissue, the site of gas gangrene infections, one of the most heavily O-glycosylated proteins is α-dystroglycan (α-DG). This protein is part of the dystrophin-glycoprotein complex (DGC) that is situated at the sarcolemma, a membrane sheet that surrounds each muscle fibre (Martin, 2003). The α-DG protein consists of three sections (Brancaccio et al., 1995), where the central region is designated as the mucin domain due to the high concentration of O-mannose and N-acetylgalactosamine (GalNAc)-initiated O-glycans (Gomez Toledo et al., 2012) (Endo, 2015). In this study, we showed that EngCP was able to hydrolyse and release the Gal-β,3GalNAc disaccharides of α-DG, as demonstrated by the absence of binding by PNA lectin. However, this hydrolysis was dependent on the presence of the sialidase, NanL. The synergism between α-DG and NanL agreed with a previous study that showed that EngCP worked co-operatively with sialidase to remove O-glycans from gastric mucin and cell surface glycoproteins of CHO 3B2A cells (Ashida et al., 2008). Similar sialidase dependence has been observed with other endo α-N-acetylgalactosaminidas (Garbe and Collin, 2012). When both EngCP and NanL were present, only a very faint α-DG band was observed, compared to the band observed when the myotube lysate was treated with NanL alone. This result suggested that most of the desialylated O-GalNAc-initiated glycans had been released by EngCP.

The effect of the removal of the O-GalNAc glycans is currently unknown, as the function of these disaccharides is unclear (Muntoni et al., 2008). Most work on the O-glycans on α-DG has been directed at O-mannose glycans in the mucin domain, since they have been shown to interact with laminin (Hara et al., 2011; Yoshida-Moriguchi and Campbell, 2015; Yoshida-Moriguchi et al., 2010). Specifically, the laminin binding site on α-DG was shown to be the core 3 M3 glycan at Thr317 (Hara et al., 2011; Yagi et al., 2013). The interaction between α-DG and laminin is important, as it establishes a critical link between the sarcolemma and the ECM, which in turn is responsible for the structural integrity of the myofibres. The results from this study indicate that EngCP does not cleave this important O-glycan. Firstly, the IIH5 antibody used in Western blotting analysis, which specifically recognises the glycosylated form of α-DG (Ervasti and Campbell, 1993), was still able to detect α-DG in the myotube lysates that had been de-O-glycosylated by EngCP. Further evidence was obtained from the laminin overlay assays. The epitope recognised by the IIH5 antibody overlaps with the laminin binding site on α-DG (Brown et al., 1999; Ervasti and Campbell, 1993). Our results showed that laminin was still able to bind to the α-DG in the EngCP-treated myotube lysates, implying that the core 3 M3 glycan was still intact. Finally, glycan array analysis showed that mannose glycans were bound to, but not cleaved by, EngCP (Table 1). Taken together these results suggest that whilst EngCP can cleave O-GalNAc glycans, it is not able to hydrolyse core M3 glycans.

The results from the in vitro analysis indicated that EngCP did not disrupt the interaction between laminin and α-DG and would therefore have little effect on myofibre integrity. To evaluate the effect of EngCP in vivo, mice were injected with EBD, a dye that has been used extensively to assess sarcolemma damage in muscular dystrophy studies (Hamer et al., 2002; Straub et al., 1997; Wooddell et al., 2010), prior to being injected with isogenic C. perfringens stains. Our results showed that EBD had entered and accumulated in the infected muscle tissues of mice injected with all C. perfringens strains, indicating the presence of myofibre membrane damage. The amount of EBD extracted from tissues infected with the engCP mutant was similar to that from animals infected with the wild-type or complemented mutant strains, suggesting that there were similar levels of membrane damage in the tissues. Overall, this finding indicated that EngCP did not contribute to myofibre damage in necrotic muscle tissue under the conditions tested, which agreed with the in vitro results.

Nonetheless, phenotypic differences were observed with the infected mice. Firstly, the engCP mutation caused the strain to be significantly attenuated for virulence. Both the 12 h virulence trials and
the 4 h EBD injection trials clearly demonstrated that disease progression was slower in the mice injected with the engCP mutant, such that each disease parameter scored lower than those of the mice injected with the wild-type and complemented mutant strains. Secondly, blue rings indicative of the accumulation of EBD were observed in the ankle area of most of the mice injected with the wild-type and engCP complemented strains but were less prevalent in the mice injected with the engCP mutant. This observation may be linked with the swelling and blackening of the footpad, since the cumulative scores for these disease parameters were lower in the mice injected with the engCP mutant compared to the mice injected with the wild type or the complemented mutant.

Although EngCP had little effect on thigh muscle membrane permeability, it clearly influenced the virulence of the organism. To investigate other potential target(s) of EngCP, glycans array analysis was carried out at two different binding temperatures to provide insight into the glycans that are bound or hydrolysed by EngCP. The results showed that EngCP recognised 23 different glycans at 4 °C, compared to only 15 glycans at 25 °C (Table 1). The analysis at 25 °C indicated that five structures containing a β1-3GalNAc motif (Glycan IDs 89, 255, 262, 362 and 17 G; Table 1) could be hydrolysed by EngCP. Even at 4 °C a wide variety of β1-3GalNAc containing glycans could not be observed on the glycan array (Table 1; Dataset S1). At this time, we do not know if this result was due to lack of recognition of these structures by EngCP, because of the array presentation, or if EngCP still had residual activity at 4 °C and the lack of binding was the result of hydrolysis of these glycans.

Binding targets identified at 4 °C included blood group antigens, Lewis antigens, P antigens, GalNAc core structures and glycosaminoglycans. Whilst many of the blood group antigens can be found on the membranes of erythrocytes, they can also be localised on the surface of many different cell types and in body fluids and secretions (Daniels, 2015). Furthermore, these antigens have been found on cells involved in the immune response, such as leukocytes and platelets (Daniels, 2013). Lewis antigens and blood group A and B antigens have been identified on lymphocytes, while the P1 antigen has been found on lymphocytes, monocytes and neutrophils (Dunstan, 1986). Blood group A and B antigens are associated with the integrin complex gp1bIIa on the surface of platelets (Hou et al., 1996; Ogusawara et al., 1993) and this complex has been implicated in the mechanism of gas gangrene disease. Activation of gp1bIIa by C. perfringens α-toxin leads to platelet-platelet and platelet-leukocyte aggregation in the vasculature (Bryant et al., 2000), ultimately resulting in leukostasis. Furthermore, gp1bIIa has been shown to be O-glycosylated, a state that is important for protein stability and function (Wang et al., 2012). Although EngCP was found to bind to these glycan structures, the effect of this O-glycosidase on these proteins, and how these interactions influence virulence remains to be elucidated.

In addition to the blood antigens, EngCP was also found to bind to GAGs such as dermatan sulfate, chondroitin-6-sulfate and hyaluronic acid. In normal muscle tissue, these GAGs surround or contour muscle fibres and blood vessels (Bertolotto et al., 1983, 1987). EngCP recognised three hyaluronic acid (or hyaluronan) structures at 4 °C. The loss of all hyaluronic acid binding by EngCP at 25 °C may indicate enzymatic hydrolysis of these structures. Hyaluronic acid is a component of the ECM and is a putative target of C. perfringens (Hynes and Walton, 2000; Matsuhashi and Okabe, 2001). Our glycan array results suggested that EngCP could potentially hydrolyse this GAG to aid in local tissue destruction by disrupting the extracellular matrix.

In conclusion, our study has demonstrated that the EngCP endo α-N-acetylgalactosaminidase is required for the full virulence of C. perfringens. This finding indicates that although the toxins produced by this organism are important, other non-toxin virulence factors also play a critical role in the disease process. The recommended treatment of clostridial myonecrosis caused by C. perfringens is surgical debridement alongside antibiotic therapy with penicillin and clindamycin (Stevens et al., 2014). Perhaps inhibitors of EngCP may have potential for the control of myonecrosis in combination with conventional antibiotic therapy.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2020.151398.

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