Host serum factor triggers germination of *Clostridium perfringens* spores lacking the cortex hydrolysis machinery

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Clostridium perfringens type A is the causative agent of a variety of histotoxic and enteric diseases. The ability of *C. perfringens* spores to germinate *in vivo* might be due to the presence of nutrient germinants in the host tissue and blood. In the current study, we investigated the ability of spores of *C. perfringens* wild-type and mutation strains to germinate in blood. Results indicate that spores of all three surveyed *C. perfringens* wild-type isolates germinated better in blood than in brain heart infusion (BHI) broth. However, as expected, spores lacking germinant receptor (GR) protein GerAA or GerKB germinated like wild-type spores in BHI broth and blood. Strikingly, while spores lacking GR proteins GerKA and GerKC showed significantly decreased germination in BHI broth, these spores germinated well in blood, suggesting that blood factor(s) can trigger spore germination through a GR-independent pathway. Using *C. perfringens* spores lacking cortex lytic enzymes (Δ*cspB* or Δ*sleC* Δ*sleM*), we were able to identify a host serum germination factor with peptidoglycan hydrolysing activity that (i) restored the colony-forming efficiencies of Δ*cspB* and Δ*sleC* Δ*sleM* spores up to ~5–20 % of that of total colony-forming spores; (ii) increased the number of c.f.u. of decoated Δ*cspB* and Δ*sleC* Δ*sleM* spores to ~99 % of that of colony-forming spores; (iii) and finally lost enzymic activity after heat inactivation, consistent with serum germination factor being an enzyme. Further characterization demonstrated that serum germination factor is very likely lysozyme, which can form a stable high molecular mass complex of ~120 kDa in serum. In conclusion, the current study indicates that a host serum germination factor with peptidoglycan hydrolysing activity is capable of triggering germination of *C. perfringens* spores by directly degrading the spore peptidoglycan cortex. Collectively, this study contributes to our understanding of the mechanism of *in vivo* germination of spores of *C. perfringens*.

INTRODUCTION

Clostridium perfringens is a Gram-positive, spore forming, anaerobic bacterium, which is highly associated with a wide array of gastrointestinal (GI) and histotoxic diseases in both humans and animals (Amimoto *et al.*, 2007; Keyburn *et al.*, 2008; McClane, 2007). However, the most common cause of *C. perfringens*-associated food poisoning in humans is the consumption of *C. perfringens* vegetative cells followed by sporulation in the gut (McClane, 2007;

Paredes-Sabja & Sarker, 2009). The early steps in the development of *C. perfringens*-associated gas gangrene and other non-food-borne GI illnesses, such as antibiotic-associated diarrhoea, occur when *C. perfringens* spores ubiquitously found in the environment come in contact with the host, where spores undergo germination followed by outgrowth, cell proliferation and toxin secretion. Therefore, *C. perfringens* spore germination can be considered as the most essential and earliest step in the progression of gas gangrene and antibiotic-associated diarrhoea.

Bacterial spores break their dormancy when they sense the presence of nutrients (termed germinants) in the environment, the germination process being triggered by germinants binding to their cognate germinant receptors (GRs) (Paredes-Sabja *et al.*, 2011; Setlow, 2003). In *C. perfringens*, the main GR involved in sensing nutrient

germinants is GerKA-KC (Paredes-Sabja *et al.*, 2008b, 2009a). Binding of the germinant to its cognate receptor leads to a variety of biophysical events, including the release of monovalent cations followed by the release of the spore's large deposit of calcium dipicolinate (Ca-DPA) (Setlow, 2003). The release of dipicolinic acid chelated at a 1 : 1 ratio with monovalent cations, predominantly Ca^{2+} (Ca-DPA), in *Bacillus subtilis* activates one of the two redundant cortex-lytic enzymes (Paidhungat *et al.*, 2001). However, in *C. perfringens*, Ca-DPA triggers spore germination through the GerKA-KC receptor (Paredes-Sabja *et al.*, 2008b). Spore peptidoglycan cortex hydrolysis in *C. perfringens* is triggered through a Ca-DPA-independent pathway (Paredes-Sabja *et al.*, 2009b, c). Degradation of the spore peptidoglycan cortex eliminates a physical constraint, allowing the spore core to uptake water to levels found in growing bacteria, and consequently to the resumption of metabolism and outgrowth (Setlow, 2003).

Besides nutrient germinants, host-specific factors also play important roles in spore germination. Previous studies have shown that *in vitro* lysozyme can trigger germination of bacterial spores, bypassing the GRs and directly degrading the spore cortex-lytic enzyme (Lund & Peck, 1994; Peck *et al.*, 1992). Indeed, in a recent study with *Bacillus anthracis* (Giebel *et al.*, 2009), it has been shown that spores lacking all cortex lytic enzymes are able to germinate *in vitro* with whole blood and serum despite their lack of germination in nutrient-rich media, and kill 25 % of the challenged mice in 4 days, suggesting that host-specific enzymes with peptidoglycan-hydrolysing activity might be involved in spore germination and virulence *in vivo*. Consequently, in this study we found that the main GR proteins, GerKA and GerKC, are not required for *C. perfringens* spore germination in blood due to the presence of a host serum germination factor with peptidoglycan hydrolysing activity (most likely lysozyme) that triggers significant germination by directly degrading the spore's peptidoglycan cortex.

METHODS

Bacterial strains. Our initial attempt in using spores of *C. perfringens* strain 13, an experimentally proven gas gangrene isolate (Awad *et al.*, 2001), failed due to its inability to sporulate under laboratory conditions. Indeed, an early stop codon was detected in the *spo0A* gene, encoding a master regulator of sporulation, in strain 13 (Huang *et al.*, 2004; Shimizu *et al.*, 2002). Therefore, we surveyed spores from three other *C. perfringens* wild-type strains: ATCC 3624 (type A, enterotoxin-negative), F4969 (non-food-borne type A, enterotoxin-positive) (Sarker *et al.*, 2000), and SM101 (electroporatable derivative of a food poisoning type A strain NCTC8798, enterotoxin-positive) (Zhao & Melville, 1998). All these wild-type isolates are capable of producing alpha toxin and thus have the potential to cause gas gangrene. SM101 mutation strain derivatives used in this study were: (i) those lacking GR-encoding genes, such as DPS101 ($\Delta gerKA$-KC), DPS103 ($\Delta gerAA$) (Paredes-Sabja *et al.*, 2008b) and DPS108 ($\Delta gerKB$) (Paredes-Sabja *et al.*, 2009a); (ii) those lacking either the cortex lytic enzymes, DPS110 ($\Delta sleC$ $\Delta sleM$) (Paredes-Sabja *et al.*, 2009c), or CspB, involved in processing of pro-SleC into mature SleC, DPS117 ($\Delta cspB$)

(Paredes-Sabja *et al.*, 2009b). Spores of strains DPS117 and DPS110 are defective in peptidoglycan cortex hydrolysis and thus also defective in colony formation on brain heart infusion (BHI) agar plates (Paredes-Sabja *et al.*, 2009b, c).

Spore preparation and purification. *C. perfringens* isolates were grown overnight in fluid thioglycollate medium (Difco; BD) at 37 °C as described by Kokai-Kun *et al.* (1994). For *C. perfringens* sporulating cultures, 10 ml Duncan Strong sporulating medium (Duncan & Strong, 1968) was inoculated with 0.2 ml fluid thioglycollate culture, and incubated overnight at 37 °C to form spores as confirmed by phase-contrast microscopy. Large amounts of spores were prepared by scaling-up the latter procedure. Spore preparations were cleaned by repeated washing with sterile distilled water and centrifugation until spores were >99 % free of sporulating cells, cell debris and germinated spores, before being resuspended in distilled water at a final OD_{600} of ~6 and stored at −20 °C (Paredes-Sabja *et al.*, 2008b).

Decoating of spores. Spore coats were extracted as previously described (Paredes-Sabja *et al.*, 2008a). Briefly, spore suspensions at an OD_{600} of ~20 were incubated in 1 ml 50 mM Tris/HCl (pH 8.0), 8 M urea, 1 % (w/v) SDS, 50 mM DTT for 90 min at 37 °C, and washed five times with 150 mM NaCl and twice with distilled water. Decoated spore suspensions were adjusted to an OD_{600} of ~6 and stored at −20 °C until use.

Germination assays. Germination of spores of *C. perfringens* SM101 wild-type and Ger⁻ mutation strains in BHI broth (Difco; BD) and sheep blood was quantified by loss of spore heat resistance. Briefly, the initial c.f.u. value was determined by plating serially diluted aliquots of heat-activated (80 °C, 10 min) spore suspensions on BHI agar plates, incubating overnight under anaerobic conditions at 37 °C and counting the colonies. To determine the germinated spore fraction in BHI broth and sheep blood, heat-activated (80 °C, 10 min) spores were resuspended in BHI broth and sheep blood to a final concentration of 10^7 c.f.u. ml⁻¹. After 60 min of incubation at 37 °C, germinating spore suspensions were subjected to wet heat at 80 °C for 20 min to kill germinated spores, and aliquots were serially diluted and plated onto BHI agar, incubated overnight at 37 °C under anaerobic conditions and the colonies counted. The germination percentage was calculated using the following formula: [1−(heat resistant c.f.u. ml⁻¹)/(initial c.f.u. ml⁻¹)] × 100.

To quantify the increase in c.f.u. counts of *C. perfringens* SM101 wild-type and Ger⁻ mutation strain spores, aliquots of heat-activated spore suspensions at an OD_{600} ~1 were directly plated onto BHI agar or incubated for 1 h at 37 °C in sheep blood, and aliquots plated onto BHI agar and incubated anaerobically overnight at 37 °C. Results were expressed as c.f.u. ml⁻¹/OD_{600} (c.f.u. ml⁻¹ at OD_{600}=1). To examine the germination of *C. perfringens* cortex lytic enzyme mutants, 50 μl spore suspension with an OD_{600} ~6 was added to 250 μl BHI broth, blood, serum or lysozyme, and incubated at 37 °C for 1–5 h. For visualization, 5 μl germinated (5 h) samples were applied to poly-L-lysine coated glass microscope slides and examined under phase-contrast microscopy. To quantify the germination, aliquots of 1 h germinated samples were serially diluted, plated onto BHI agar, incubated overnight at 37 °C under anaerobic conditions and the number of colonies counted. The colony-forming efficiency of untreated spores, expressed as c.f.u. ml⁻¹/OD_{600}, was compared to those of decoated spores plated on BHI agar containing 1 μg lysozyme ml⁻¹.

Serum filtration and inactivation. Sheep serum was obtained by centrifugation (3000 *g*, 10 min) of defibrinated sheep blood (PML Microbiological). Sheep serum and human serum (HS) (BioWhittaker; Lonza) were fractionated into low molecular mass (LMM) (<30 kDa) and high molecular mass (HMM) (>30 kDa) fractions by centrifugation (4000 *g*, 15 min) using centrifugal filters

with a nominal molecular mass limit of 30 kDa (Amicon Ultra-4) and stored at 4 °C until use. Heat-inactivated serum was prepared by heating sheep serum and HS at 57 °C for 60 min, which was cooled on ice and stored at 4 °C until use.

SDS-PAGE and Western blotting. Serum fractions were analysed with SDS-PAGE. Briefly, 1 μl untreated sheep serum and HS, 10 and 5 μl LMM serum fraction and 1 μl HMM serum fractions of sheep serum and HS were boiled with an equal volume of SDS-PAGE loading buffer (125 mM Tris-HCL, 20 % glycerol, 4 % SDS, 0.01 % bromophenol blue, 2 % 2-mercaptoethanol) and run on SDS-PAGE (18 % acrylamide) gels, and the gels were stained with Coomassie brilliant blue (Bio-Rad Laboratories).

For Western blotting, monoclonal anti-human lysozyme antibody (Abcam) and polyclonal anti-lysozyme antibody (Invitrogen) were used. Since the monoclonal anti-human lysozyme antibody epitope is disulfide bond dependent (Abcam), samples were mixed with SDS-PAGE loading buffer in the absence of β-mercaptoethanol and heat treatment. Proteins were run on SDS-PAGE (12 % acrylamide) gels and transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore). These Western blots were probed with a 1 : 500 dilution of mouse monoclonal anti-human lysozyme antibody (Abcam) for 24 h at room temperature and then with 1 : 10 000 dilution of goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Promega) for 1 h at room temperature, in TBS (25 mM Tris base, pH 7.4/150 mM NaCl) with 4 % BSA and 0.05 % Tween. HRP activity was detected with a chemiluminescence detection system (Molecular Imager ChemiDoc XRS+ System; Bio-Rad) by using the PicoMax sensitive chemiluminescent HRP substrate (Rockland Immunochemicals). Each Western blot also included 10 μl PageRuler multicolour broad range protein ladder (Fermentas).

RESULTS

Blood triggers germination of spores of *C. perfringens* wild-type and GR mutation strain isolates

Spores of all three surveyed *C. perfringens* wild-type strains, irrespective of their isolation source, germinated well in blood (Fig. 1). However, BHI-triggered spore germination varied between strains; the highest germination was observed with SM101, followed by F4969 and ATCC3624, as measured by ~95, 50 and 25 % germination, respectively (Fig. 1). When spores of SM101 derivatives with mutations in the GR-encoding genes (i.e. Δ*gerKA-KC*, Δ*gerKB* and Δ*gerAA* spores) were assayed, the majority of Δ*gerAA* and Δ*gerKB* spores germinated with either BHI broth or sheep blood (Fig. 1). Surprisingly, while only ~50 % of Δ*gerKA-KC* spores germinated in BHI broth, incubation of Δ*gerKA-KC* spores in sheep blood significantly (P<0.005) increased germination to >90 % (Fig. 1). These results indicate that at least GerKA-KC is not required for sheep blood-triggered germination of *C. perfringens* spores, and suggest that an additional germination factor(s) present in the sheep blood might be triggering spore germination through a GR-independent pathway.

Host serum germination factor possesses peptidoglycan hydrolysing activity

To evaluate if factor(s) in sheep blood can trigger *C. perfringens* spore germination by directly degrading the
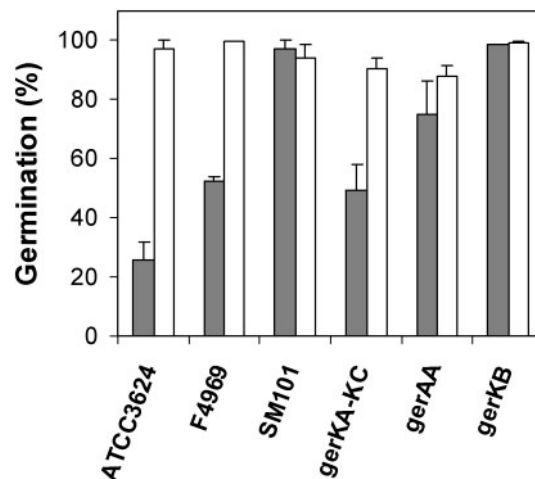

Fig. 1. Germination of *C. perfringens* spores in blood does not require GerKA-KC. Heat-activated spores of *C. perfringens* strains ATCC3624 (wild-type), SM101 (wild-type), F4969 (wild-type), DPS101 (Δ*gerKA-KC*), DPS103 (Δ*gerAA*) and DPS108 (Δ*gerKB*) were incubated at 37 °C for 60 min in BHI broth (grey bars) or sheep blood (white bars), and germination was measured by monitoring the loss of spore heat resistance as described in Methods.

spore peptidoglycan cortex, the germination of spores lacking cortex lytic enzymes were assayed. As expected, Δ*cspB* and Δ*sleC* Δ*sleM* spores incubated in BHI broth prior to plating onto BHI agar resulted in a ~100 000-fold lower c.f.u. value than seen when decoated Δ*cspB* and Δ*sleC* Δ*sleM* spores were directly plated onto BHI agar containing lysozyme (Fig. 2). Strikingly, when Δ*cspB* and Δ*sleC* Δ*sleM* spores were incubated in sheep blood prior to plating onto BHI agar, a significant increase (P<0.001) in c.f.u. counts was observed (Fig. 2). The Δ*cspB* and Δ*sleC* Δ*sleM* spores yielded similar c.f.u. counts both in whole blood and serum (Fig. 2); however, c.f.u. counts of Δ*cspB* spores incubated in HS were significantly higher than for those incubated in sheep serum and the opposite effect was found with Δ*sleC* Δ*sleM* spores (Fig. 2). Collectively, these results indicate that the germination factor is present in the serum of sheep and humans, and this serum germination factor possesses enzymic activity that can directly degrade the spore peptidoglycan cortex.

Spore coats are known to provide some protection from exogenous cortex hydrolysing enzymes (Klobutcher *et al.*, 2006; Laaberki & Dworkin, 2008). Therefore, if the serum germination factor possesses peptidoglycan hydrolysing activity it should increase the germination efficiency of Δ*cspB* and Δ*sleC* Δ*sleM* spores upon removal of their coats. Some indirect evidence came from the observation that serum germination factor triggered germination in only ~10 % of total colony-forming Δ*cspB* and Δ*sleC* Δ*sleM* spores (Fig. 2), suggesting that the serum germination factor was able to trigger germination presumably of coat-defective Δ*cspB* and Δ*sleC* Δ*sleM* spores. We first compared
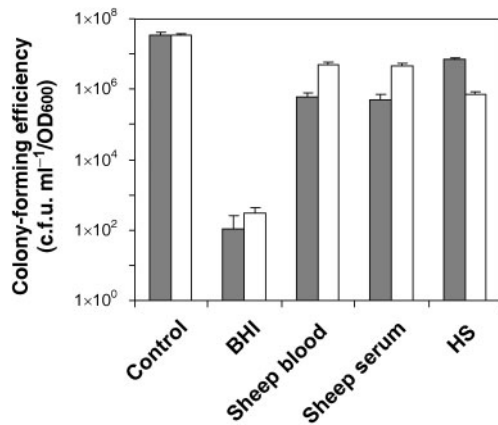
Fig. 2. Blood and serum increases colony-forming efficiency of *C. perfringens* spores lacking cortex lytic enzymes. Heat-activated spores of *C. perfringens* strains DPS117 (Δ*cspB*) (grey bars) and DPS110 (Δ*sleC* Δ*sleM*) (white bars) were incubated in BHI broth, blood serum at 37 °C for 60 min, and colonies were counted after plating on BHI agar. As a control for the maximum colony-forming efficiency of spores of strains DPS117 and DPS110, decoated spores were heat activated, plated onto BHI agar containing lysozyme and the colonies counted.

the germination efficiency of decoated and untreated Δ*cspB* spores under phase-contrast microscopy. As expected, similar fractions of untreated Δ*cspB* spores became phase dark when incubated with HS and lysozyme for 5 h (Fig. 3). Decoating Δ*cspB* spores increased phase dark spores to >99 % at 5 h incubation in either HS or lysozyme (Fig. 3), suggesting that *C. perfringens* spore coats provide some

protection against the peptidoglycan cortex hydrolytic activity of the serum germination factor.

Next, germination experiments were repeated with untreated and decoated Δ*cspB* and Δ*sleC* Δ*sleM* spores, and the number of c.f.u. was counted. Untreated Δ*cspB* and Δ*sleC* Δ*sleM* spores incubated with lysozyme or HS yielded a higher number of c.f.u. than those directly plated onto BHI plates (Fig. 4). However, when decoated Δ*cspB* and Δ*sleC* Δ*sleM* spores were germinated with HS, c.f.u. levels were restored to those of decoated spores plated directly onto BHI agar with lysozyme (Fig. 4). Similar results were obtained when these experiments were repeated with sheep serum (data not shown). Collectively, these results indicate that spore coats act as a physical barrier for serum factor-triggered germination, and provide evidence that the serum germination factor has peptidoglycan hydrolysing activity that degrades the spore peptidoglycan cortex.

Serum germination factor's peptidoglycan hydrolysing activity is heat labile and present in the HMM fraction of serum

The heat stability of the serum germination factor was evaluated by heat inactivating (57 °C for 60 min) sheep serum and HS, and assaying for germination of Δ*cspB* and Δ*sleC* Δ*sleM* spores. The Δ*cspB* and Δ*sleC* Δ*sleM* spores incubated with heat-inactivated HS did not show an increase in their c.f.u. counts, which were similar to those with BHI broth (Fig. 5). Similar results were obtained with heat-inactivated sheep serum (data not shown). These results indicate that the serum germination factor's peptidoglycan hydrolysing activity is heat labile, which is
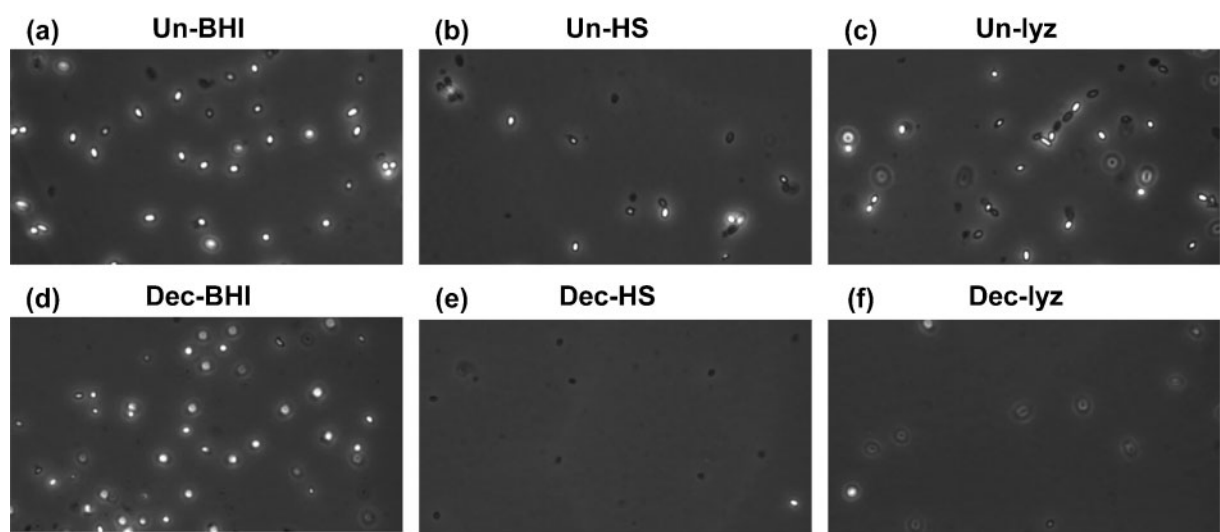

Fig. 3. HS increases germination of decoated Δ*cspB* spores. Untreated (a, b, c) and decoated (d, e, f) spores of strain DPS117 (Δ*cspB*) were heat activated and incubated at 37 °C for 5 h in BHI broth (a, d), HS (b, e) and 1 µg lysozyme ml^{-1} (c, f), and then photographed under phase-contrast microscopy.
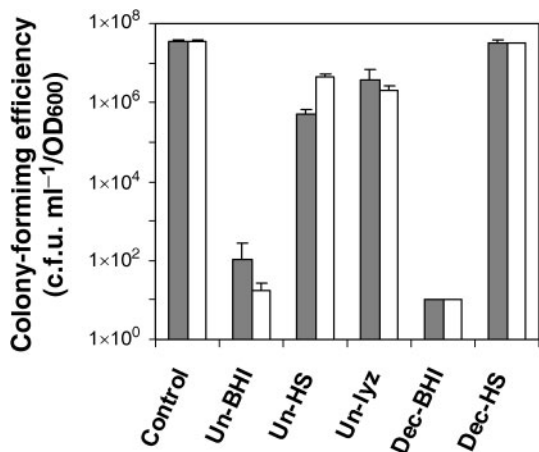
Fig. 4. HS increases colony-forming efficiency of decoated $\Delta cspB$ and $\Delta sleC$ $\Delta sleM$ spores. Untreated or decoated spores of strains DPS117 ($\Delta cspB$) (grey bars) and DPS110 ($\Delta sleC$ $\Delta sleM$) (white bars) were heat activated and incubated at 37 °C for 60 min with BHI or HS, plated onto BHI agar and the colonies counted. Un-BHI, untreated spores in BHI broth; un-HS, untreated spores in HS; dec-BHI, decoated spores in BHI broth; dec-HS, decoated spores in HS; and control, decoated spores that were heat activated and plated onto BHI containing lysozyme.
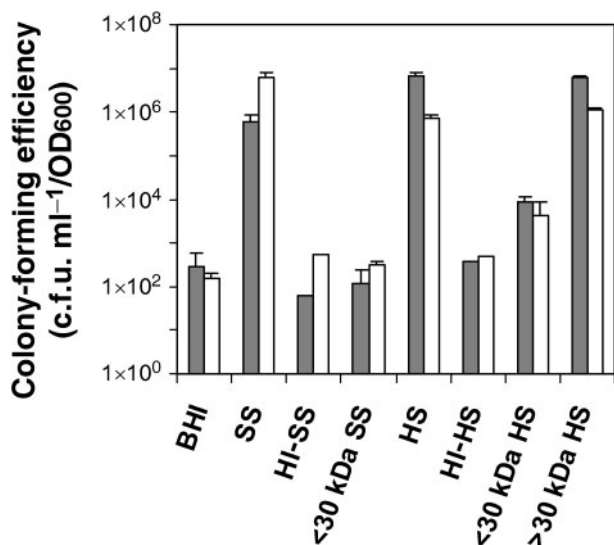

Fig. 5. Serum germination factor has a molecular mass >30 kDa. Heat-activated spores of *C. perfringens* strains DPS117 ($\Delta cspB$) (grey bars) and DPS110 ($\Delta sleC$ $\Delta sleM$) (white bars) were incubated at 37 °C for 60 min with: BHI broth, sheep serum (SS), heat inactivated sheep serum (HI-SS), LMM sheep serum (<30 kDa SS), HS, heat inactivated HS (HI-HS), LMM HS (<30 kDa HS) or HMM HS (>30 kDa HS). The c.f.u. counts were determined by plating onto BHI agar.

consistent with serum germination factor being an enzyme in nature (Fig. 4).

In an attempt to separate the protein with peptidoglycan hydrolysing activity, sheep serum and HS were fractionated by ultrafilter centrifugation with a filter with a molecular cut-off mass of 30 kDa. The filtered serum expected to contain species of a molecular mass <30 kDa, hereafter LMM, was used for germination (Fig. 5). Sheep serum-LMM was unable to induce germination of $\Delta cspB$ and $\Delta sleC$ $\Delta sleM$ spores; i.e. similar levels of c.f.u. were observed with sheep serum-LMM and BHI broth. Although slight increases in c.f.u. counts for $\Delta cspB$ and $\Delta sleC$ $\Delta sleM$ spores were observed with HS-LMM, these were significantly ($P<0.05$) lower than that with unfiltered HS (Fig. 5). When $\Delta cspB$ and $\Delta sleC$ $\Delta sleM$ spores were germinated in the serum fraction of a molecular mass of >30 kDa (HMM), similar c.f.u. levels as for unfiltered sheep serum and HS were observed (Fig. 5; data not shown). These results suggest that the serum germination factor's peptidoglycan hydrolysing activity is present in the HMM fraction of serum.

Detection of lysozyme in the HMM fraction of serum

In mammals, lysozyme is the only protein with peptidoglycan hydrolysing activity identified to date (Prager & Jolles, 1996), and early studies have reported antimicrobial lysozyme activity with a molecular mass of ~16 kDa in HS (Inoue *et al.*, 1959; Michael & Braun, 1964). However, the serum is compromised by a 'network' of protein/peptide and protein–protein interactions, and removal of the high-abundance serum carrier proteins also removes the low-abundance peptides and proteins (Zhou *et al.*, 2004). This suggests that the serum germination factor, likely serum lysozyme, might be interacting with other serum proteins forming a HMM complex that cannot pass through the 30 kDa filter membrane.

To investigate possible interactions of the serum germination factor (lysozyme) with HMM carrier proteins, HS, HMM and LMM were analysed by SDS-PAGE and Western blotting with monoclonal anti-human lysozyme antibody. While no LMM-specific protein band was detected on a SDS-PAGE gel, a smear of protein bands of >30 kDa was found in the HMM fraction (Fig. 6a). Strikingly, Western blotting under non-reducing conditions detected a ~120 kDa band immunoreactive to lysozyme mAb in HS and HMM (Fig. 6b). However, no immunoreactive band was detected in the LMM fraction of HS (Fig. 6b). These results were confirmed using a polyclonal anti-lysozyme antibody with essentially similar results (data not shown). Interestingly, a free lysozyme specific band with a predicted molecular mass of ~16 kDa was missing in the HMM fraction (Fig. 6b). Collectively, these results indicate that lysozyme is present in the serum forming a stable complex with HMM proteins, and is absent in the LMM fraction, suggesting that the serum germination factor with spore peptidoglycan hydrolysis activity might be serum lysozyme.
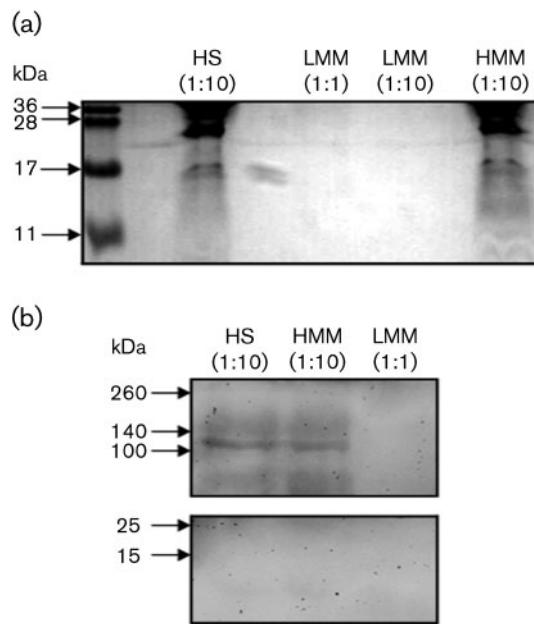
Fig. 6. Lysozyme forms a HMMC in HS. (a) HS was fractionated with a 30 kDa cut-off molecular mass filter and run in a SDS-PAGE gel. HS and the >30 kDa fraction (HMM) were mixed at a 1:10 ratio with SDS sample buffer, while the <30 kDa fraction (LMM) was mixed at a 1:1 or 1:10 ratio and loaded in an 18% SDS-PAGE gel. The gel was electrophoresed at 100 V for 1–2 h and stained with Coomassie brilliant blue. (b) Aliquots of HS, HMM and LMM fractions were separated under non-reducing conditions by SDS-PAGE, proteins transferred to a PVDF (polyvinylidene fluoride) membrane and probed with lysozyme mAb as described in Methods. The arrows indicate the relative locations of known molecular mass proteins.

DISCUSSION

The ability of *C. perfringens* spores to germinate, outgrow and return to vegetative cells with high speed (Paredes-Sabja *et al.*, 2009a) seems to provide great advantage for this anaerobic bacterium in the development of gas gangrene and other non-food-borne GI illnesses, such as antibiotic-associated diarrhoea. Despite the good understanding of the pathogenesis of *C. perfringens*-mediated gas gangrene and other non-food-borne illnesses, there is little knowledge available on the molecular basis involved in the early stages (i.e. *in vivo* spore germination and outgrowth) of these diseases. In this context, our present study offers several significant contributions. First, all surveyed *C. perfringens* spores germinated well in blood and the main GR proteins, GerKA and GerKC, are not required for this blood-triggered germination. It was surprising that while only ~50% of Δ*gerKA-KC* spores germinated with BHI, ~90% did so when incubated with sheep blood serum, indicating that serum germination factor significantly increased germination of Δ*gerKA-KC* spores. The implications of these findings are that spores with defective GRs are still able to germinate inside the host, and that endogenous serum germination factor might be promoting *in vivo* germination. However, it is not yet clear how *C. perfringens* spores germinate *in vivo* without heat activation (80 °C, 10 min), a process known to increase the commitment of spores to germinate possibly through a GR-dependent pathway (Stewart *et al.*, 1981; Yi & Setlow, 2010; Zhang *et al.*, 2010).

A second major conclusion offered by this work is that the host serum germination factor is likely to be serum lysozyme that triggers *C. perfringens* spore germination by directly degrading the spore peptidoglycan cortex. Indeed, the fact that *C. perfringens* spores with defects in the major components of the cortex lytic machinery were able to significantly germinate and show increased c.f.u. counts when incubated with serum, suggests that the serum germination factor has peptidoglycan hydrolysing activity. To our knowledge, the only host enzyme with peptidoglycan hydrolysing activity identified to date is lysozyme (Hankiewicz & Swierczek, 1974; Pier *et al.*, 2004; Prager & Jolles, 1996; Reitamo *et al.*, 1978), which hydrolyses the bacterial cell wall through cleavage of the linkage bond of *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues of the peptidoglycan (Cole & Ganz, 2005). Our Western blot analyses using monoclonal anti-human lysozyme antibody detected lysozyme-specific immunoreactive bands in HS and HMM fractions, but not in the LMM fraction, results that are consistent with the peptidoglycan hydrolysis activity observed in HS and HMM fractions. Our finding, that the serum germination factor is indeed lysozyme, was consistent with results from some early studies where purified lysozyme triggered germination of heat-injured and coat-deficient *C. perfringens* spores (Cassier & Sebald, 1969; Cassier & Ryter, 1971; Sacks, 1981). However, our current study advanced our understanding of the mechanism of lysozyme-triggered spore germination by showing that serum lysozyme triggered germination of *C. perfringens* spores by bypassing the GRs and directly degrading the spore peptidoglycan cortex.

A third and perhaps the most important conclusion from this work is that the host's lysozyme forms a high molecular mass complex (HMMC) in the serum, as a lysozyme-specific band of ~120 kDa, but not a free lysozyme-specific band of ~16 kDa, was detected only in HMM fractions of serum. It is possible that the lysozyme-like proteins (of molecular masses of 14–16 kDa) might be interacting with more abundant protein species present in the serum, forming a HMMC incapable of passing through the 30 kDa cut-off filter. Although a previous study (Zhou *et al.*, 2004) successfully fractionated a LMM fraction (<30 kDa) from HS using a YM-30 Centricon ultrafiltration device, the sample was previously boiled, followed by other treatments, in contrast to the native conditions used in this study. An obvious question is whether this HMMC would enhance the ability of lysozyme to interact with pathogenic bacteria. Indeed, while the majority of untreated Δ*cspB* spores became phase dark (indicating full spore core hydration) when incubated with HS, the majority of untreated Δ*cspB* spores became phase grey (indicating partial spore core hydration)

when treated with lysozyme. In contrast, serum and purified lysozyme were equally efficient in decoated $\Delta cspB$ spores. In this context, although there are many other components in the serum that might enhance spore germination triggered by serum lysozyme, it might be possible that the serum HMMC might facilitate spore–lysozyme interaction in the presence of spore coats and also perhaps increase lysozyme's peptidoglycan hydrolysing activity. However, further studies will be needed to prove this.

ACKNOWLEDGEMENTS

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