Adherence of *Clostridium difficile* spores to Caco-2 cells in culture

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*Clostridium difficile* is the causative agent of the majority of antibiotic associated diarrhoea cases. *C. difficile* spores are recognized as the persistent and infectious morphotype as well as the vehicle of transmission of CDI. However, there is a lack of knowledge on how *C. difficile* spores interact with the host’s epithelial surfaces. In this context, we have characterized the ability of *C. difficile* spores to adhere to human Caco-2 cells. Despite the similarities in spore-surface hydrophobicity between spores of *C. difficile* and *Clostridium perfringens* (another enteric pathogen that also sporulates in the gut), spores of *C. difficile* adhere better to Caco-2 cells. Adherence to Caco-2 cells was significantly reduced when *C. difficile* spores were treated with trypsin. Sonication of *C. difficile* spores altered the ultrastructure of the outermost exosporium-like structure, releasing two protein species of ~40 kDa and significantly reduced spore hydrophobicity and adherence to Caco-2 cells. Using a trifunctional cross-linker, we were able to co-immunoprecipitate four protein species from the surface of Caco-2 cells. In conclusion, this study provides evidence that *C. difficile* spores adhere to human intestinal enterocyte-like cells through spore- and enterocytic-surface-specific ligand(s) and/or receptor(s).

INTRODUCTION

*Clostridium difficile* is a Gram-positive, anaerobic, spore-forming enteric pathogenic bacterium and is the causative agent of pseudomembranous colitis and 15–20% of antibiotic associated diarrhoea cases (Viswanathan et al., 2010). *Clostridium difficile* infection (CDI) is a significant cause of morbidity and is responsible for economic losses of ~4 billion dollars annually in the United States and European Union (Kuijper et al., 2006). Antibiotic treatments disrupt the normal intestinal microbiota, allowing *C. difficile* spores to germinate and outgrow native bacteria, colonizing the host’s intestinal tract, causing CDI and secreting toxins. Two toxins, TcdA and TcdB, have been identified as the major virulence factors in *C. difficile* pathogenesis (Kuehne et al., 2010; Lyras et al., 2009; Voth & Ballard, 2005). They act as glycosyltransferases and modify small GTPases of the Rho family of proteins within the host cell, causing alterations in the cytoskeleton (Auwex, 1991; Voth & Ballard, 2005). A third toxin, named CTD toxin, is produced by few strains; it also plays a role in pathogenesis and increases adherence of *C. difficile* cells to the host’s epithelial surfaces (Schwan et al., 2009). However, the complexity of CDI symptoms suggests that other less-studied non-toxin-related virulence factors and traits might be involved in *C. difficile* pathogenesis.

Although recent studies suggest that *C. difficile* epidemic strains do not have an exceptionally high sporulation rate (Akerlund et al., 2008; Merrigan et al., 2010; Sirard et al., 2011), there is a general consensus that *C. difficile* spores are the persistent and infectious morphotype as well as the vehicle of transmission of CDI. During the course of clinical infections, an increase in numbers of *C. difficile* spores has been observed in the stools (Deneve et al., 2009; Hookman & Barkin, 2009; McFarland, 2005), indicating that *C. difficile* sporulates inside the host, leading to persistence of *C. difficile* spores in the intestinal tract. This is supported by the high levels of sporulation of *C. difficile* observed in the intestinal tracts of hamster models (Goulding et al., 2009). The persistence of *C. difficile* spores in the colon of CDI-patients complicates effective CDI treatments since *C. difficile* spores exhibit resistance to all currently available treatments (McFarland, 2005) and can therefore survive in the colon following CDI treatments. Interestingly, CDI has a relatively high rate (20%) of relapse (Pepin et al., 2005), with ~25–85% of the relapse cases being attributed to the initial strain (Barbut et al., 2000; O’Neill et al., 1991; Oka et al., 2012), suggesting that some strains of *C. difficile* might have spores with unique properties that allow them to adhere to the host’s intestinal epithelial surfaces.
C. difficile vegetative cells adhere to specific components of the extracellular matrix (ECM) such as fibrinogen, laminin, fibronectin and collagen I, III and IV (Cerquetti et al., 2002). Studies have shown that C. difficile vegetative cells bind to intestinal epithelial cells and interact with apical microvilli of differentiated Caco-2 cells (Cerquetti et al., 2002; Evellard et al., 1993). Several C. difficile cell-surface proteins have been shown to play a role in adherence to intestinal epithelial cells: (i) the cell-surface protein Cwp66, with adhesive properties (Waligora et al., 2001); (ii) the fibronectin-binding protein Fbp68 (Hennequin et al., 2003); (iii) S-layer proteins (Calabi et al., 2002); and (iv) the flagella, composed of the flagellin FlIC and the flagellar cap protein FlICα, which are involved in mucus attachment (Tastery et al., 2001). However, to the best of our knowledge, there is a lack of information on the adherence of C. difficile spores to intestinal epithelial cells. Therefore, in this study we have characterized the adherence of C. difficile spores to cultured Caco-2 cells, an intestinal epithelial cell line previously employed to characterize in vitro adherence of C. difficile vegetative cells to the intestinal epithelium (Drudy et al., 2001; Evellard et al., 1993; Naaber et al., 1996). This study describes the attachment of C. difficile spores to Caco-2 cells. Using biotinylation of Caco-2 cell-surface proteins we were able to detect specific proteins of Caco-2 cells that interact with C. difficile spores. Furthermore, we show that two specific C. difficile spore-proteins might be involved in spore adherence.

**METHODS**

**Bacterial strains, human cell lines and chemicals.** C. difficile strains 630 (tcdA+, tcdB+, tcdC+, cdtA+, cdtB), Pitt 51 (tcdA+, tcdB+, tcdC+, cdtA+, cdtB+) and Pitt 177 (tcdA+, tcdB+, tcdC+, cdtA+, cdtB+) (McEllistrem et al., 2005; Paredes-Sabja & Sarker, 2011); Clostridium perfringens strains P4969 and SM101; and Bacillus subtilis strain PS832 (Collie & McClane, 1998; Paidhungat et al., 2001; Waters et al., 2003) were used in this study and have been described elsewhere. C. difficile strains Pitt51 and Pitt177 were isolated from patients presenting clinical symptoms of CDI in a tertiary care hospital in Pittsburgh, USA (McEllistrem et al., 2005). Caco-2 cells were grown in Eagle’s minimal essential medium (EMEM) (BioWhittaker, Lonza) and used between passages 50 and 80. Medium was supplemented with 20% (v/v) fetal-calf serum (ATCC), penicillin (100 μg ml⁻¹) and streptomycin (100 μg ml⁻¹).

**Spore preparation and purification.** C. difficile spores were prepared as described previously (Paredes-Sabja & Sarker, 2011; Sorg & Sonenshein, 2008). Briefly, C. difficile strains were grown in Brain Heart Infusion broth (Difco) supplemented with 0.5% yeast extract (Difco) (BHIS) anaerobically at 37 °C. Next, overnight cultures in BHIS medium were diluted to an OD₆₀₀ of 0.2 and plated onto BHIS agar and incubated under anaerobic conditions at 37 °C overnight. For total C. difficile spores, spore-infected Caco-2 cells wells were not washed and Caco-2 cells were directly lysed with 100 μl 0.06% Triton X-100 for 30 min at 37 °C, plating onto Brain Heart Infusion agar supplemented with 0.5% yeast extract, 2% glucose and 0.1% sodium taurocholate (BHIS+ST) (Himedia Laboratories), and incubated under anaerobic conditions at 37 °C overnight. For total C. difficile spores, spore-infected Caco-2 cells wells were not washed and Caco-2 cells were directly lysed with 100 μl 0.06% Triton X-100 for 30 min at 37 °C and the lysed spore-infected Caco-2 cells were plated directly onto BHIS+ST agar plates and incubated anaerobically overnight at 37 °C. Number of c.f.u. ml⁻¹ was determined and percentage of adherence was calculated using the following formula: (final c.f.u. ml⁻¹/initial c.f.u. ml⁻¹) × 100.

**Adherence assay.** To measure adherence of viable C. difficile and C. perfringens spores, Caco-2 cells were seeded (8 × 10⁵ cells per well) onto 24-well plates and incubated for 5 days to a final density of 1 × 10⁸ cells per well. Prior to adherence, Caco-2 cells were washed three times with Dulbecco’s PBS (DPBS) (BioWhittaker, Lonza) and infected with C. difficile spores at an m.o.i. of 4 or 10 in 200 μl of EMEM. Spore-infected Caco-2 cells were incubated for 1 h at 37 °C under aerobic conditions. To remove unbound C. difficile spores from spore-infected Caco-2 cells, the wells were washed three times with DPBS. Caco-2 cells were then lysed with 100 μl 0.06% Triton X-100 for 30 min at 37 °C, plated onto Brain Heart Infusion agar supplemented with 0.5% yeast extract, 2% glucose and 0.1% sodium taurocholate (BHIS+ST) (Himedia Laboratories), and incubated under anaerobic conditions at 37 °C overnight. For total C. difficile spores, spore-infected Caco-2 cells wells were not washed and Caco-2 cells were directly lysed with 100 μl 0.06% Triton X-100 for 30 min at 37 °C and the lysed spore-infected Caco-2 cells were plated directly onto BHIS+ST agar plates and incubated anaerobically overnight at 37 °C. Number of c.f.u. ml⁻¹ was determined and percentage of adherence was calculated using the following formula: (final c.f.u. ml⁻¹/initial c.f.u. ml⁻¹) × 100.

**Alexa Fluor 488-labeling of C. difficile and C. perfringens spores.** Purified spores were labelled with Alexa Fluor 488 and biotin as described previously (Agerer et al., 2004) with minor modifications. Briefly, purified C. difficile and C. perfringens spores (~3 × 10⁸ spores) were washed with 0.1 M sodium bicarbonate (pH 8.2) and resuspended in 300 μl 0.1 M sodium bicarbonate (pH 8.2) and 0.02 mg ml⁻¹ Alexa 488 carboxylic acid, TFP ester, bis(triethylammonium salt) reactive dye (Molecular Probes, Invitrogen) and incubated for 45 min at room temperature. Alexa Fluor 488-labelled spores were counted with a Heber Bacteria Counting Chamber Z30 (Hawksley) and stored at −20 °C until use.

C. perfringens spores were prepared as described previously (Paredes-Sabja & Sarker, 2011). Briefly, overnight cultures of C. perfringens isolates grown in fluid thioglycollate (Difco) medium were inoculated into Duncan–Strong (DS) sporulation medium (Duncan & Strong, 1968) and incubated at 37 °C for 24 h. Pure spore suspensions were obtained by repeated centrifugation and resuspension with sterile distilled water until spore suspensions were >99% clean of sporulating cells, cell debris and germinated spores, and stored at −20 °C until use.

Spores of Bacillus subtilis were prepared by growing cultures at 37 °C for 96 h on BHI agar plates as described previously (Nicholson & Setlow, 1990) and purified as described above.

**Adherence assay.** To measure adherence of viable C. difficile and C. perfringens spores, Caco-2 cells were seeded (8 × 10⁵ cells per well) onto 24-well plates and incubated for 5 days to a final density of 1 × 10⁸ cells per well. Prior to adherence, Caco-2 cells were washed three times with Dulbecco’s PBS (DPBS) (BioWhittaker, Lonza) and infected with C. difficile spores at an m.o.i. of 4 or 10 in 200 μl of EMEM. Spore-infected Caco-2 cells were incubated for 1 h at 37 °C under aerobic conditions. To remove unbound C. difficile spores from spore-infected Caco-2 cells, the wells were washed three times with DPBS. Caco-2 cells were then lysed with 100 μl 0.06% Triton X-100 for 30 min at 37 °C, plated onto Brain Heart Infusion agar supplemented with 0.5% yeast extract, 2% glucose and 0.1% sodium taurocholate (BHIS+ST) (Himedia Laboratories), and incubated under anaerobic conditions at 37 °C overnight. For total C. difficile spores, spore-infected Caco-2 cells wells were not washed and Caco-2 cells were directly lysed with 100 μl 0.06% Triton X-100 for 30 min at 37 °C and the lysed spore-infected Caco-2 cells were plated directly onto BHIS+ST agar plates and incubated anaerobically overnight at 37 °C. Number of c.f.u. ml⁻¹ was determined and percentage of adherence was calculated using the following formula: (final c.f.u. ml⁻¹/initial c.f.u. ml⁻¹) × 100.

Adherence of C. difficile and C. perfringens spores was also quantified by fluorescent microscopy. Briefly, Caco-2 cells were seeded (4 × 10⁵ cells per well) onto 8-well culture slides (FD Falcon) and incubated for up to 5 days to a final density of 7 × 10⁵ cells per well. Prior to infection, confluent Caco-2 cells were washed three times with DPBS and infected using 100 μl of EMEM containing Alexa Fluor 488-labelled C. difficile and C. perfringens spores at an m.o.i. of 4 or 10 and incubated under aerobic conditions for 1 h at 37 °C in a 5% CO₂ atmosphere. Incubation for up to 2 h did not significantly increase the ability of C. difficile spores to adhere to Caco-2 cells (data not shown). Wells were then washed three times with DPBS to remove unbound labelled spores and fixed with 200 μl freshly prepared 4% paraformaldehyde for 15 min at room temperature and washed twice with DPBS. Next, cells were permeabilized with 0.06% Triton X-100 in DPBS for 15 min at 37 °C, washed twice with DPBS, stained for F-actin with 1 U of Alexa Fluor 568-phalloidin conjugate (Molecular Probes, Invitrogen) for 30 min and rinsed three times with DPBS. To evaluate the effect of EDTA on adherence of C. difficile spores to Caco-2 cells, 5-day-old Caco-2 cell monolayers were pretreated with...
Ca²⁺-free DPBS (Lonza) containing 0.1 mM EDTA tetrasodium salt (Versene, Lonza) for 1 h prior to infection in a 5 % CO₂ atmosphere at 37 °C. The cells were subsequently infected at an m.o.i. of 4 with C. difficile 630 spores in DPBS/EDTA for 1 h at 37 °C in a 5 % CO₂ atmosphere and treated as described above. Samples were air-dried and mounted with Cytoseal 60 (Thermo Scientific) on cover slides and sealed with nail polish. A Leica DM400B fluorescence microscope was used to quantify total extra- and intra-cellular C. difficile and C. perfringens spores adhered to Caco-2 cells. Photomicrographs were prepared with Adobe Photoshop and Microsoft Picture Manager Software.

**Hydrophobicity assay.** Relative hydrophobicity of spores was measured by the bacterial adherence to hydrocarbon (BATH) method (Brahmbhatt et al., 2007; Rosenberg et al., 1980). Briefly, C. difficile and C. perfringens spores were resuspended in sterile distilled water to a final OD₄₅₀ = 0.5 and mixed with the non-aqueous solvent hexadecane (Merck). Adherence to hydrocarbons was measured by loss of turbidity in the aqueous phase. A ratio of 0.1 ml hydrocarbon ml⁻¹ spore suspension yielded sufficient separation (data not shown), which corresponds to a 567 mL hexadecane concentration. Suspensions were vortexed for 30 s and the phases were allowed to separate for 15 min at room temperature. Loss of turbidity of the aqueous solution was measured and the hydrophobicity of the spore’s surface was calculated by the following formula: 100−(final OD₄₄₀/initial OD₄₄₀) x 100.

**Trypsin treatment of C. difficile spores.** C. difficile spores (×10⁸) were incubated with trypsin (2.5 mg ml⁻¹) in 25 mM PBS (pH 7.8) for 3 h at room temperature. Trypsin-treated C. difficile spores were washed five times with 150 mM NaCl and twice with distilled water and stored at −20 °C until use.

**Decoating and sonication of C. difficile spores.** Spore coats were extracted as described previously (Paredes-Sabja et al., 2008). Briefly, C. difficile spores (×10⁸) were incubated in 1 ml of 50 mM Tris/HCl (pH 8.0) containing 8 M urea, 1 % (w/v) SDS and 50 mM dithiothreitol for 90 min at 37 °C. Decoated C. difficile spores were washed five times with 150 mM NaCl and twice with distilled water before being stored at −20 °C until use.

C. difficile spores were sonicated to remove the outermost surface layers as described previously (Kang et al., 2005). Briefly, C. difficile spores were resuspended in 50 mM Tris/HCl and 0.5 mM EDTA buffer (pH 7.5). All subsequent manipulations were done in ice-cold conditions. C. difficile spores were sonicated (Microson Ultrasonic Lab, Fisher Scientific) with a 1 cm diameter probe for 1 h at 20 °C. The sonicated suspension was described. The supernatant was concentrated by vacuum centrifugation and analysed by Western blot.

To determine the amount of total biotinylated proteins remaining in the sonicated spores, sonicated spores were also analysed by Western blot using a 1:10,000 dilution of streptavidin conjugated with IRDye 680 (Licor). Band density of blots was quantified with ImageJ Software (http://rsb.info.nih.gov/ij/index.html).

**Electron microscopy.** For scanning electron microscopy (SEM), samples were fixed with 2.5 % glutaraldehyde and 1 % paraformaldehyde in 0.1 M cacodylate buffer and serially dehydrated for 20 min each with 30, 50, 75 and 90 % acetone followed by two final dehydration steps of 100 % acetone for 20 min. Dehydrated samples were subjected to critical point drying and coated with gold and palladium and analysed with an FEI Quanta 600PEM scanning electron microscope. For transmission electron microscopy (TEM), untreated, sonicated and decoated C. difficile spores were applied to glow discharge carbon-coated grids for negative staining in 1 % (w/v) phosphotungstic acid. Grids were then washed extensively to remove fixative and negatively stained with 1 % uranyl acetate. Alternatively, to identify the effect of sonication on other structural features of C. difficile spores, untreated and sonicated C. difficile spores were fixed with freshly prepared 2.5 % glutaraldehyde and 1 % paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4 °C. Secondary fixation was performed with 1 % osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2). Samples were then rinsed in cacodylate buffer, stained for 30 min with 1 % tannic acid and rinsed off with cacodylate buffer. Samples were dehydrated with a step-wise acetone gradient of 30 % (stained with 2 % uranyl acetate at this stage) for 30 min, 50 % for 30 min, 70 % overnight, 90 % for 30 min and twice with 100 % acetone. Dehydrated samples were embedded in Spurr’s resin at ratios of 3:1, 1:1 and 1:3 (acetone : Spurr’s) for 40 min each, resuspended in 100 % Spurr’s resin for 4 h and baked overnight at 65 °C. Thin sections were obtained using a microtome and were placed on glow discharge carbon-coated grids for negative staining and double lead stained with 2 % uranyl acetate and lead citrate. Samples were examined at 80 kV with a Philips EM300 transmission electron microscope in the Electron Microscopy Facility at Oregon State University and with a Phillips Tecnai 12 Bio Twin at the Electron Microscopy facility at Pontificia Universidad Catolica de Chile.

**Syto Green fluorescent labelling of decoated C. difficile spores.** Since decoated C. difficile spores were inefficiently labelled with Alexa Fluor 488, primarily because primary amine groups required by the TFP esters present in Alexa dyes for labelling might have been efficiently removed during decoating treatment, decoated C. difficile spores were labelled with 10 μM Syto Green Fluorescent Nucleic Acid Stain (Molecular Probes, Invitrogen) in PBS for 3 h at 4 °C and labelling efficiency was evaluated by fluorescence microscopy. Syto-labelled spores were counted with a Heber Bacteria Counting Chamber Z300 (Hawksley) and stored at −20 °C until use.

**Photo cross-linking biotin-label transfer assay.** Labelling of putative surface receptors of Caco-2 cells was performed using a biotin-labelled trifunctional cross-linking reagent as described previously (Oliva et al., 2008). Briefly, C. difficile spores (5 × 10⁸ spores) were incubated with 1 ml 0.1 M bicarbonate buffer (pH 8.2) containing 2 mg Sulfo-SBED (Pierce) for 30 min at room temperature. Conjugated C. difficile spores were washed three times with DPBS, quantified as described above and stored at −80 °C until use. Labelling reactions were carried out in the dark. Next, the conjugated C. difficile spores were added at an m.o.i. of 50 to cells in Petri dishes and incubated at 37 °C for 1 h in the dark. Infected Caco-2 cells were washed twice to eliminate unbound conjugated spores and subsequently treated with UV light at 365 nm (XX-15B lamp; Spectrolite) at a distance of 6 cm for 20 min at 4 °C. Cells were lysed in DPBS containing 0.1 % Tween and 0.4 % Triton X-100 in the presence of protease inhibitors (Promega), the disulfide bond of the trifunctional cross-linker was reduced with 100 mM DTT and Caco-2 cell biotin-labelled surface.
proteins were analysed by Western blot using 1:10 000 streptavidin conjugated with IRDye 680 (Licor).

Statistical analyses. All experiments were carried out in duplicate and repeated at least three times. In some experiments, Student’s t test was used to compare specific treatments and \( P<0.05 \) was considered significant using the statistical software Statgraphics Centurion XVI (StatPoint Technologies).

RESULTS

**C. difficile** spores exhibit higher levels of adherence to cultured Caco-2 cells than **C. perfringens** and **B. subtilis** spores

Preliminary experiments of adherence of **C. difficile** 630 spores under aerobic and anaerobic conditions gave essentially the same results (data not shown). Therefore, all subsequent experiments were conducted under aerobic conditions. First, we evaluated if **C. difficile** spores had a greater ability to adhere to Caco-2 cells than spores of other spore-forming species. As a negative control, we used spores of **B. subtilis**, as this micro-organism does not colonize the intestinal tract. We incubated **B. subtilis** P832 spores with Caco-2 cells under aerobic conditions and the fraction of adhered spores was quantified by viable counts. Unexpectedly, nearly 50 % of **B. subtilis** spores adhered to monolayers of Caco-2 cells (Fig. 1a), while nearly 70 % of **C. difficile** spores of strain 630 adhered to monolayers of Caco-2 cells (Fig. 1a). Spores of **C. difficile** strains Pitt51 and Pitt177 exhibited slightly lower levels of adherence compared to spores of strain 630 (Fig. 1a). For comparison, we used spores of **C. perfringens**, another anaerobic enteric pathogen whose spores lack the exosporium-like structure (Novak et al., 2003; Orsburn et al., 2008). **C. perfringens** spores of two gastrointestinal disease-related isolates (SM101 and F4969) had significantly lower levels of adherence to monolayers of Caco-2 cells than those of **C. difficile** spores (Fig. 1a).

To confirm the above results, spores of **C. difficile** 630 and **C. perfringens** F4969 were fluorescently labelled prior to infecting Caco-2 cells. The results indicated that **C. difficile** 630 spores adhered well to monolayers of Caco-2 cells and exhibited significantly (\( P<0.01 \)) higher levels of adherence than fluorescently labelled **C. perfringens** F4969 spores (Fig. 1b). Collectively, these results indicate that **C. difficile** spores exhibit higher levels of adherence to monolayers of Caco-2 cells than **C. perfringens** spores.

To evaluate if **C. difficile** spores also adhere better to the cell periphery of Caco-2 cells, monolayers of Caco-2 cells were pretreated with the Ca\(^{2+}\)-chelating agent (EDTA) and subsequently infected with fluorescently labelled **C. difficile** 630 spores. Notably, **C. difficile** spores adhered significantly (\( P<0.01 \)) better to EDTA-treated than to untreated monolayers of Caco-2 cells (Fig. 2a–c). Adherence of **C. difficile** 630 spores to untreated monolayers of Caco-2 cells was targeted to the whole apical surface of Caco-2 cells (Fig. 2b), whereas **C. difficile** spores mainly adhered to the periphery of EDTA-treated Caco-2 cells (Fig. 2c). These results indicate that **C. difficile** spores adhere better to the periphery than to the apical surface of 5-day-old monolayers of EDTA-treated Caco-2 cells.

**Spore surface hydrophobicity is involved in adherence of **C. difficile** but not **C. perfringens** spores**

Hydrophobic interactions have been shown to be involved in the adherence of *Bacillus cereus* spores to Caco-2 cells (Andersson et al., 1998). Therefore, to evaluate whether the
observed adherence would correlate with spore surface hydrophobicity, we measured the percentage of removed spores with 567 mM hexadecane. Interestingly, the \textit{C. difficile} spores of strains with lower levels of adherence to \textit{Caco-2} cells (Pitt51 and Pitt177) (Fig. 1a) had significantly (\(P<0.01\)) lower spore surface hydrophobicity than spores of strain 630, which displayed higher levels of adherence to \textit{Caco-2} cells (Fig. 1a and Fig. 3), suggesting that hydrophobicity of the spore surface plays a role in adherence of \textit{C. difficile} spores to epithelial cells. However, despite the lower levels of adherence observed with \textit{C. perfringens} spores, these spores had similar levels of hydrophobicity than \textit{C. difficile} strain 630 spores (Fig. 1a, b and Fig. 3). Strikingly, \textit{B. subtilis} spores had significantly (\(P<0.01\)) lower levels of hydrophobicity than spores of \textit{C. difficile} and \textit{C. perfringens} strains (Fig. 3). Collectively, these results suggest that (i) \textit{C. difficile} spores with higher levels of hydrophobicity have greater ability to adhere to \textit{Caco-2} cells, (ii) \textit{C. perfringens} spores have different surface proteins and/or different amounts of similar surface proteins than those found in \textit{C. difficile} spores that promote adherence to \textit{Caco-2} cells, and (iii) \textit{B. subtilis} adherence to \textit{Caco-2} cells does not require hydrophobic interactions.

**Trypsin treatment reduces adherence of \textit{C. difficile} spores to the apical surface of \textit{Caco-2} cells**

In order to test the hypothesis that \textit{C. difficile} spore-surface proteins are involved in adherence of \textit{C. difficile} spores to monolayers of \textit{Caco-2} cells, \textit{C. difficile} 630 spores were trypsinized and assayed for adherence. No reduction in spore viability was observed in trypsin-treated spores (data not shown). Interestingly, when \textit{Caco-2} cells were infected with trypsin-treated \textit{C. difficile} spores, an approximately fourfold reduction in adherence of viable spores was observed when

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**Fig. 2.** Effect of EDTA on adherence of \textit{C. difficile} spores to \textit{Caco-2} cells. (a) Monolayers of 5-day-old \textit{Caco-2} cells were pretreated with Ca\(^{2+}\)-free DPBS-0.1 mM EDTA prior to infection as chelation of Ca\(^{2+}\) with EDTA disrupts the intercellular junctions. EDTA-treated \textit{Caco-2} cells were subsequently infected with fluorescently labelled \textit{C. difficile} spores at an m.o.i. of 4. The number of adhered fluorescently labelled \textit{C. difficile} 630 spores was determined by fluorescent microscopy. Asterisks denote statistical difference (\(P<0.01\)) between control and EDTA-treated cells. (b, c) Representative fluorescent micrographs of untreated (b) and EDTA-treated (c) 5-day-old \textit{Caco-2} cell monolayers showing spore adhesion to the apical surface and periphery of \textit{Caco-2} cells, respectively.

**Fig. 3.** Comparison of hydrophobicity of \textit{C. difficile}, \textit{C. perfringens} and \textit{B. subtilis} spores. Hydrophobicity of spores of \textit{C. difficile} strains 630, Pitt51 and Pitt177, \textit{C. perfringens} strains F4969 and SM101, and \textit{B. subtilis} strain PS832 was measured with 567 mM hexadecane. Data represent the mean of at least three independent experiments. Error bars represent SEM. *Statistical difference (\(P<0.01\)) when \textit{B. subtilis} P832 was compared to \textit{C. difficile} strain 630 or \textit{C. perfringens} strains SM101 or F4969. **Statistical difference (\(P<0.01\)) when strains Pitt51, Pitt177, F4969 and SM101 are compared with \textit{C. difficile} strain 630.
compared to untreated C. difficile spores (data not shown), suggesting that C. difficile spore-surface proteins, which are removed, at least in part, by trypsin treatment, are involved in adherence to Caco-2 cells. To gain more insight into the topology of C. difficile spore adherence to enterocyte-like epithelial cells, Caco-2 cells were infected with untreated (Fig. 4a, b) and trypsin-treated (Fig. 4c, d) C. difficile 630 spores and analysed by SEM. Interestingly, SEM showed that C. difficile spores adhered evenly to the surface of Caco-2 cells (Fig. 4a). Although there was a significant reduction in the ability of trypsin-treated C. difficile spores to adhere to Caco-2 cells, the fraction that did adhere did so evenly to the surface of Caco-2 cells (Fig. 4c). Indeed, trypsin-treated spores that adhered to Caco-2 cells seemed to adhere to the microvilli using remnants of surface proteins normally involved in adherence, similar to untreated spores (Fig. 4b, d). Jointly, these results provide evidence that C. difficile spores bind to enterocyte-like epithelial cells and that spore-surface proteins are involved in this adherence.

Sonication alters spore ultrastructure by removing spore-surface proteins

In Bacillus anthracis, sonication has been reported to remove the exosporium (Kang et al., 2005). Recently, Lawley et al. (2009) demonstrated that C. difficile spores have a premature exosporium that lacks the hair-like nap typical of several Bacillus species. Indeed, our TEM of negatively stained C. difficile spores shows the outer-most layer as a translucent layer with an absence of a hair-like nap structure (Fig. 5a). Analysis of osmium tetroxide-stained untreated C. difficile 630 spores revealed that surrounding the spore coat is a diffuse outer layer, likely the exosporium-like structure, that differs significantly from the hair-like nap observed in several Bacillus species (Henriques & Moran, 2007), confirming previous observations (Lawley et al., 2009). This exosporium-like structure surrounds another electron-dense layer (Fig. 5a), this being the spore coat, with typical laminations that resemble the striated outer coats of B. subtilis spores (Henriques & Moran, 2007). To evaluate if sonication would remove the exosporium-like structure in C. difficile spores, C. difficile spores were sonicated and analysed by TEM. Interestingly, osmium tetroxide-stained sonicated spores lack the majority of the diffuse outer layer (Fig. 5a). Negative-stained sonicated spores lacked the outer-most diffuse translucent layer (data not shown), indicating that the majority of the diffuse layer surrounding the coats of C. difficile spores can be removed by sonication.

To identify what proteins were removed by sonication, C. difficile spore-surface proteins were biotinylated and subjected to either sonication or a decoating treatment as a control for outer spore protein layer removal. As expected, the decoating treatment removed the majority of the spore material surrounding the spore peptidoglycan cortex (Fig. 5b), presumably the coats and the exosporium-like structure, with little biotinylated material remaining in decoated spores as shown by Western blot analysis (data not shown). In contrast, sonication treatment only released two protein species of ~40–45 kDa that were detectable by Western blot analysis in the supernatant fraction of the sonicated biotin-labelled spores (Fig. 5b). The highest and lower molecular mass protein species represented ~72 ± 8 and 23 ± 8 % of the removed material, respectively (Fig. 5b). This suggests that these two protein species might be major components of the outer-most diffuse layer or exosporium-like structure. It is likely that these proteins

![Fig. 4. Scanning electron micrographs of C. difficile spores adhered to Caco-2 cells. Five-day-old Caco-2 cell monolayers were infected at an m.o.i. of 100 with untreated (a, b) and trypsin-treated (c, d) C. difficile 630 spores and analysed by SEM. The micrographs show that untreated, and to a lesser extent trypsin-treated, C. difficile spores interact with immature microvilli (white arrows) on the apical surfaces of Caco-2 cell monolayers.](http://jmm.sgmjournals.org)
were also present in coat extracts; however, their presence might be covered by more abundant proteins of similar size (Fig. 5b). Interestingly, the hydrophobicity of sonicated spores was significantly lower than that of untreated spores (Fig. 5c), suggesting that the protein species of ~40–45 kDa that were removed by sonication play a role in the hydrophobicity of C. difficile spores. Collectively, these results suggest that the structural properties of the C. difficile spore exosporium might be significantly different than that of B. anthracis.

Decoating and sonication reduces adherence of C. difficile spores to Caco-2 cells

Since at least some of the ~40–45 kDa protein species were removed in sonicated C. difficile 630 spores and certainly all protein from decoated spores, we hypothesized that these sonicated and decoated spores would have reduced adherence to monolayers of Caco-2 cells. Fluorescent microscopy analyses demonstrated that few, if any, decoated C. difficile 630 spores were able to adhere to monolayers of Caco-2 cells (Fig. 6). Adherence of sonicated C. difficile 630 spores to monolayers of Caco-2 cells was significantly reduced compared to untreated spores (Fig. 6). Collectively, these results support the hypothesis that sonication removed some proteins (e.g. species of ~40–45 kDa) that have a role in adherence of C. difficile spores to monolayers of Caco-2 cells.

Pull-down of putative receptor(s) of Caco-2 cells

In an attempt to identify potential candidate proteins in Caco-2 cells that might be recognized by C. difficile spores as spore-specific receptor(s), we labelled C. difficile 630 spores with a heterobifunctional cross-linker and infected 5-day-old monolayers of Caco-2 cells. Proteins directly interacting with C. difficile spores were cross-linked by UV-light activation of the aryl azide group and the biotin group was transferred to the unknown proteins by cleaving the disulfide bond. Biotinylated receptor(s) from spore-free lysates of biotinylated Caco-2 cells were pulled down with streptavidin-IRDye 680 conjugate. Strikingly, four major protein species with molecular masses ranging from ~26 to 40 kDa were detectable (Fig. 7). There was variation in the relative abundance of these proteins as determined by densitometry.
The adherence of *Clostridium difficile* spores to the intestinal mucosa may be the first stage of CDI and could play a significant role in persistence of *C. difficile* spores in healthy individuals as well as in CDI relapse episodes. In this context, our results provide evidence that *C. difficile* spores are able to adhere to enterocyte-like epithelial cells and suggest that this adherence is mediated by one or more spore- and host-specific proteins. Since Caco-2 cells resemble small intestinal enterocyte-like cells, these findings might have implications regarding the pathogenesis of *C. difficile* in the adherence of ingested *C. difficile* spores to the small intestine, increasing the reservoir of *C. difficile* spores throughout the digestive tract.

The spore surface of *Bacillus* species (i.e. *B. anthracis* and *B. cereus*) is characterized by an exosporium structure with the presence of a hair-like nap that surrounds the basal layer of the exosporium (Henriques & Moran, 2007). Although it is suggested that *C. difficile* spores possess an exosporium-like structure (Lawley et al., 2009), the features of this structure are significantly different to that of the *B. anthracis* and *B. cereus* exosporium (Henriques & Moran, 2007). In this context, our TEM results are in agreement with previous findings (Lawley et al., 2009) and highlight the absence of a hair-like nap, suggesting that the diffuse-layer that surrounds *C. difficile* spores might be structurally and functionally different than that of *B. anthracis* and *B. cereus* (Henriques & Moran, 2007). Sonication of *C. difficile* spores provides evidence supporting these differences. Sonication treatment typically strips off the exosporium fraction in *B. anthracis* spores which is composed of ~20 proteins (Henriques & Moran, 2007); however, only two *C. difficile* proteins of ~40–45 kDa were efficiently extracted when biotinylated *C. difficile* spores were subjected to sonication. Absence of these two protein species significantly affected the ultrastructure of the exosporium-like diffuse outermost layer of *C. difficile* spores. It was most striking that sonicated spores had reduced hydrophobicity and lower levels of adherence to Caco-2 cells than that of untreated *C. difficile* spores. Collectively, these results suggest that the two ~40–45 kDa spore-specific proteins could be directly involved in the adherence of *C. difficile* spores to Caco-2 cells and that the nature of such an interaction might be related to hydrophobicity. It is tempting to hypothesize that the lower levels of adherence to monolayers of Caco-2 cells and lower levels of hydrophobicity seen in 567 mM hexadecane-treated *C. difficile* Pitt51 and Pitt177 spores, as compared to *C. difficile* 630 spores, could be attributed to the lower abundance of both ~40–45 kDa protein species on the surface of *C. difficile* Pitt51 and Pitt177 spores. The
decoating treatment of *C. difficile* spores readily stripped off of their outer layers, presumably the coats and exosporium-like layer, similar to the effect seen with *B. subtilis* and *C. perfringens* spores. Few, if any, of these decoated spores adhered to Caco-2 cells, even to a lesser extent than sonicated spores, indicating that either sonication did not remove the ~40–45 kDa proteins involved in adherence completely or there are other spore-surface proteins that have auxiliary roles in adherence of *C. difficile* spores to Caco-2 cells.

Although the adherence of *C. difficile* vegetative cells was not tested in this study, it has been reported previously that ~1% of total cells adhere to Caco-2 cells (Dingle *et al.*, 2010, 2011), which is much lower than the ~50–70% adherence of *C. difficile* spores and supports the hypothesis that spore adherence might be exploited by *C. difficile* as a means of persistence in the host. Despite the fact that the colonic environment is mostly anaerobic, there is an oxygen concentration gradient from the colonic lumen to the colonic epithelium. The experimental conditions used in this study, although acceptable for *C. difficile* spores, are not recommended for *C. difficile* spore outgrowth studies as germinated *C. difficile* spores are rapidly inactivated by the presence of oxygen. Trypsin treatment of *C. difficile* spores showed that adherence was dependent on spore-surface proteins that were cleaved by trypsin, suggesting that *C. difficile* spores also possess specific proteins that promote attachment to the apical surface of Caco-2 cells and in absence of these proteins, binding becomes non-specific. In addition to the apical cell surface, the cell periphery might be a relevant site of adherence of *C. difficile* spores to the intestinal epithelial cells, presumably during the course of CDI. However, the increase of adherence to EDTA-treated cells, although significant, was small and might not hold biological relevance. Co-immunoprecipitation experiments demonstrated that *C. difficile* spores interact specifically with proteins on the surface of apical microvilli of Caco-2 cells, strongly supporting the hypothesis that *C. difficile* spores adhere to specific receptor protein(s) on the surface of the apical microvilli of Caco-2 cells. Work in our laboratories seeks to identify and characterize these putative *C. difficile* spore receptor(s).

*C. difficile* and *C. perfringens* are both anaerobic spore-forming enteric pathogens; however, the mechanism of pathogenesis of these bacteria is significantly different (Denève *et al.*, 2009; Paredes-Sabja & Sarker, 2009; Uzal & McClane, 2011). Indeed, the lesser ability of *C. perfringens* spores to adhere to monolayers of Caco-2 cells, compared to *C. difficile* spores, indicates that, in contrast to *C. difficile*, adherence of *C. perfringens* spores to the intestinal epithelium is not a significant aspect of its pathogenesis, although this might be the case for *C. difficile*. It is worth noting that, although the spore surface of both species has a relatively similar level of hydrophobicity, *C. perfringens* spores adhered less to Caco-2 cells. The opposite was true for *B. subtilis* spores, which, despite having lower levels of spore-surface hydrophobicity, adhered to Caco-2 cells at similar levels as *C. difficile* spores. *B. subtilis* has been shown to be able to sporulate in the intestinal tract but it does not colonize the intestinal tract (Tam *et al.*, 2006). However, these similar levels of adherence to Caco-2 cells of spores of *B. subtilis* and *C. difficile* suggest that either *B. subtilis* spores are indeed able to adhere to the intestinal epithelium or that the adherence assay might not be a direct indicator of the ability of spores to adhere to the intestinal epithelium, meaning this experimental assay might require further improvement. Collectively, these results highlight significant differences in the spore surface of these three species and the differential nature of the interactions that promote adherence, where, at least for *C. difficile* spores, adherence is modulated by the presence of two protein species of 40–45 kDa that contribute to spore surface hydrophobicity. Further studies will be required to identify the proteins in spores of *B. subtilis* and *C. perfringens* that cause these differences.

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### REFERENCES


