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

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Running Title: *Clostridium difficile* spore adherence to Caco-2 cells

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Abstract

Clostridium difficile is the causative agent of the majority of antibiotic associated diarrheas. *C. difficile* spores are recognized as the morphotype of transmission, infection and persistence. 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 *C. 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 trypsin-treated. Sonication of *C. difficile* spores altered the ultrastructure of the outermost exosporium-like structure, released two protein species of ~ 40-kDa and significantly reduced spore-hydrophobicity and adherence to Caco-2 cells. Using a trifunctional crosslinker, we were able to co-immunoprecipitate four protein species from the surface of Caco-2 cells. In conclusion, this study provides, for the first time evidence that *C. difficile* spores adhere to human intestinal enterocyte-like cells through spore- and enterocytic-surface specific ligand(s) and/or receptor(s).

Key Words: *C. difficile*, *C. perfringens*, Spores, Adherence.

INTRODUCTION

Clostridium difficile is a Gram-positive, anaerobic, spore-forming enteric pathogenic bacterium, and the causative agent of pseudomembranous colitis and of 15 – 20% of antibiotic associated diarrhea (Viswanathan *et al.*, 2010). *Clostridium difficile* infections (CDI) are a significant cause of morbidity and 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 the germinating *C. difficile* spore to outgrow and colonize the host's intestinal tract, secreting toxins, and causing CDI. 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 Rho protein family within the host cell, producing alterations in the cytoskeleton (Auwerx, 1991; Voth & Ballard, 2005). A third toxin, named CTD toxin is produced by few strains, also plays a role in pathogenesis and increases adherence of *C. difficile* cells to host's epithelial surfaces (Schwan *et al.*, 2009). However, the complexity of CDI symptoms suggests that other less-studied non-toxin 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 consent 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 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 also supported by

high levels of sporulation of *C. difficile* observed in the intestinal tract of a hamster model (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 until suppression of CDI treatments. Interestingly, CDI has a relatively high rate (20%) of relapse (Pepin *et al.*, 2005), with ~ 25 to 85 % of the relapse 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 to adhere to the host's intestinal epithelial surfaces.

C. difficile vegetative cells adhere to the specific components of the extracellular matrix (ECM) such as fibrinogen, laminin, fibronectin, collagen I, III and IV (Cerquetti *et al.*, 2002). Studies (Cerquetti *et al.*, 2002; Eveillard *et al.*, 1993) have shown that *C. difficile* vegetative cells bind to intestinal epithelial cells, and interact with apical microvilli of differentiated Caco-2 cells (Eveillard *et al.*, 1993). Several *C. difficile* cell surface proteins have been shown to play a role in adherence to intestinal epithelial cells: i) a cell-surface protein (Cwp66) with adhesive properties (Waligora *et al.*, 2001); ii) a fibronecting-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 FliD, involved in mucus attachment (Tasteyre *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 intestinal epithelium (Drudy *et al.*, 2001; Eveillard *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 cells' 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*$^+$, *ctdA*$^-$ and *ctdB*$^-$), Pitt 51 (*tcdA*$^+$, *tcdB*$^+$, *tcdC*$^+$, *ctdA*$^+$ and *ctdB*$^+$), Pitt 177 (*tcdA*$^+$, *tcdB*$^+$, *tcdC*$^+$, *ctdA*$^+$ and *ctdB*$^+$) (McEllistrem *et al.*, 2005; Paredes-Sabja & Sarker, 2011), *C. perfringens* strains F4969 and SM101, and *Bacillus subtilis* strain PS832 are described elsewhere (Collie & McClane, 1998; Paidhungat *et al.*, 2001; Waters *et al.*, 2003). *C. difficile* strains Pitt51 and Pitt177 were isolated from patients presenting clinical symptoms of CDI in a tertiary care hospital in Pittsburg, U.S.A. (McEllistrem *et al.*, 2005). Caco-2 cells were gown in eagle's minimal essential medium (EMEM) (BioWhittaker, Lonza, Walkersville, MD) and used between passages 50 and 80. Media was supplemented with 20% (v/v) fetal-calf serum (ATCC, Manassas), penicillin (100 µg mL^{-1}) and streptomycin (100 µg mL^{-1}).

Spore preparation and purification. Preparation of *C. difficile* spores was done as described (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 BHIS cultures were diluted to an OD_{600} of 0.2 and plated onto BHIS agar and incubated under anaerobic conditions at 37°C for 7 days (Paredes-Sabja & Sarker, 2011; Sorg & Sonenshein, 2008). Sporulating cultures were recovered by flooding the plates with ice-cold sterile distilled water, and collected by centrifugation. Cell pellets were washed 10 times in ice cold water by repeated centrifugation and resuspension. Next, free spores were separated using 50% HistoDenz and washed five times to eliminate traces

of HistoDenz and until spore suspensions were > 99% free of vegetative cells, sporulating cells and cell debris. Spore suspensions were stored at -20 °C until use.

C. perfringens spores were prepared as described (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 of 96 h at 37°C on BHI agar plates as described (Nicholson & Setlow, 1990), and the spores were purified as described above.

Alexa Fluor 488-labeling of *C. difficile* and *C. perfringens* spores. Purified spores were labeled with Alexa Fluor 488 and biotin as described (Agerer *et al.*, 2004) with minor modifications. Briefly, purified *C. difficile* and *C. perfringens* spores (3×10^9 spores) were washed with 0.1 M sodium bicarbonate (pH 8.2) and resuspended in 300 µl of 0.1 M sodium bicarbonate (pH 8.2)-0.02 mg mL^{-1} of Alexa 488 carboxylic acid TFP ester, bis (triethylammonium salt) (Molecular Porbes, Invitrogen, U.S.A.) and incubated for 45 min at room temperature. Alexa Fluor 488-labeles spores were counted with a Heber Bacteria Counting Chamber Z30 (Hawksley, UK) and stored at – 20°C until use.

Adherence assay. To measure adherence of viable *C. difficile* and *C. perfringens* spores, Caco-2 cells were seeded (8×10^5 cells/well) onto 24 well plates and incubated for 5 days to a final density of ~ 1.1×10^6 cells/well. Prior to adherence, Caco-2 cells were washed there times with

Dulbecco's phosphate buffered saline (DPBS) (BioWhittaker, Lonza, Walkersville, MD), and infected with *C. difficile* spores at a multiplicity of infection (MOI) 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. Next, to remove unbound *C. difficile* spores from spore-infected Caco-2 cells, wells were washed three times with DPBS and 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 (BHISG + ST) (Himedia Laboratories Pvt. Ltd. Mumbai, India), and incubated under anaerobic conditions at 37°C overnight. For total *C. difficile* spores, spore-infected Caco-2 cells wells were not washed and were directly lysed with 100 µl 0.06% Triton X-100 for 30 min at 37°C and lysed spore-infected Caco-2 cells directly plated onto BHIISG + ST agar plates, incubated anaerobically overnight at 37°C, and colony forming units (CFU) mL^{-1} counted. Percentage of adherence was calculated using the following formula: [(Final CFU mL^{-1})/(Initial CFU mL^{-1})] x 100.

Adherence of *C. difficile* and *C. perfringens* spores was also quantified by fluorescent microscopy. Briefly, Caco-2 cells were seeded (4×10^5 cells/well) onto 8-well culture slides (FD Falcon) and incubated up to 5 days to a final density of 7×10^5 cells/well. Prior to infection, confluent Caco-2 cells were washed three times with EMEM, and infected with 100 µl of EMEM containing Alexa Fluor 488-labeled *C. difficile* and *C. perfringens* spores at an MOI of 4 or 10 and incubated under aerobic conditions for 1 h at 37°C in a 5% CO_2 atmosphere. Incubation 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 washed three times with DPBS to remove unbound labeled *C. difficile* spores, and fixed with 200 µl of 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 and stained for F-actin with 1 U of Alexa Fluor 568-phalloidin conjugate (Molecular Probes, Invitrogen, CA) 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 Ca^{2+}-free DPBS (Lonza)-0.1 mM EDTA tetrasodium salt (Versene, Lonza) for 1 h prior to infection in a 5% CO_2 atmosphere at 37°C, and subsequently infected at an MOI of 4 with *C. difficile* 630 spores in DPBS-0.1 mM EDTA tetrasodium salt (Versene, Lonza) for 1 h at 37°C in a 5% CO_2 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. DM4008B fluorescent 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 hydrophobicities of spores were 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 re-suspended in sterile distilled water to a final OD_{440} ~ 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 of hydrocarbon mL^{-1} of spore suspension yield sufficient separation (data not shown), which corresponds to a 567 mM hexadecane. Suspensions were vortexed for 30 s, and phases allowed to separate for 15 min at room temperature. Loss of turbidity of the aqueous solution was measured and the hydrophobicity of spore's surface was calculated by the following formula: $100 - [(\text{Final } OD_{440}) / (\text{Initial } OD_{440}) \times 100]$.

Trypsin treatment of *C. difficile* spores. *C. difficile* spores (~ 10^9) were incubated with trypsin (2.5 mg/ml) in 25 mM Phosphate buffered saline (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 in -20°C until use.

Decoating and Sonication of *C. difficile* spores. Spore coats were extracted as described (Paredes-Sabja *et al.*, 2008). Briefly, *C. difficile* spores (~ 10^9) were incubated in 1 ml of 50 mM Tris-HCl (pH 8.0)-8 M urea-1% (wt/vol) sodium dodecyl sulfate-50 mM dithiothritol for 90 min at 37°C. Decoated *C. difficile* spores were washed five times with 150 mM NaCl and twice with distilled water and stored in -20°C until use.

C. difficile spores were sonicated to remove the outermost surface layers as previously described (Kang *et al.*, 2005). Briefly, *C. difficile* spores were re-suspended in 50 mM Tris-HCl, 0.5 mM EDTA buffer (pH 7.5). All subsequent manipulations were done in ice-cold conditions. *C. difficile* spores were sonicated (Microson Ultrasonic Cell Disruptor XL, Misinox Incorporated, Farmingdale, NY)) with maximum power (20 Watts) for 10 1-min burst, separated by 3 min of cooling on ice. Sonicated spores were collected by centrifugation and washed three times to eliminate loosely outermost surface layers. Sonicated spores were quantified with a Heber Bacteria Counting Chamber Z300 (Hawksley, UK) and stored at -20°C until use. Sonication had no effect on viability of *C. difficile* spores (data not shown).

In other experiments, *C. difficile* spores (4 x 10^9 spores) were labeled with 1 ml of 0.1 M Bicarbonate buffer (pH 8.2) containing 2 mg of Biotinamido Hexanoic Acid N-Hydroxysuccimide Ester (Sigma-Aldrich) for 60 min at room temperature, and washed five

205 times with DPBS by centrifugation (13,200 x *g* for 10 min) to remove excess of biotin-labeling
206 reagent. Next, conjugated spores were subjected to decoating treatment as described above,
207 decoated spores were pelleted and the supernatant containing the coat/exosporium fractions and
208 the decoated spore pellet were analyzed by Western blot with streptavidin conjugated with
209 IRDye 680 (Licor, Canada) with an Odyssey Infrared Imaging System (Licor, Canada).
210 Similarly, to analyze the protein fraction released by sonication, biotin-labeled *C. difficile* spores
211 were sonicated as described above, and the supernatant fraction was concentrated by vacuum
212 centrifugation and analyzed by Western blot. To determine the amount of total biotinylated
213 proteins remaining in the sonicated spores, sonicated spores were also analyzed by Western Blot
214 using 1:10,000 dilution of streptavidin conjugated with IRDye 680 (Licor, Canada). Band
215 density of blots were quantified with ImageJ Software (http://rsb.info.nih.gov/ij/index.html).
216
217 **Electron microscopy.** For SEM, samples were fixed with 2.5% glutaraldehyde-1%
218 paraformaldehyde in 0.1 M cacodylate buffer, and serially dehydrated for 20 min each with 30%,
219 50%, 75% and 90% of acetone followed by twice with 100% for 20 min. Dehydrated samples
220 were subjected to critical point drying and coated with gold and palladium and analyzed with a
221 FEI Quanta 600PEG. For TEM, untreated, sonicated and decoated *C. difficile* spores were
222 applied to glow discharge carbon-coated grids for negative staining in 1% (wt/vol)
223 phosphotungstic acid. Grids were then washed extensively to remove fixative and negatively
224 stained with 1% uranyl acetate. Alternatively, to identify the effect of sonication on other
225 structural features of *C. difficile* spores, untreated and sonicated *C. difficile* spores were fixed
226 with freshly prepared 2.5% glutaraldehyde-1% paraformaldehyde in 0.1 M cacodylate buffer (pH
227 7.2) for overnight at 4°C. Secondary fixation was performed with 1% osmium tetroxide-0.1M

cacodylate buffer (pH 7.2), rinsed in cacodelyte 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 spurs at a ratio of acetone:spurs of 3:1, 1:1 and 1:3 for 40 min each, resuspended in 100% spurs resine for 4 h and baked overnight at 65°C. Thin sections were obtained using a micotomb and were placed on a glow discharge carbon-coated grids for negative staining and double lead stained with 2% uranyl acetate and lead citrate. Samples were evaluated at 80 kV with a Philips EM300 TEM at the Electron Microscopy Facility at Oregon State University and with a Phillips Tecnai 12 Bio Twin at the Electron Microscopy facility at Pontificia Universdad Catolica de Chile.

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

Photo cross-linking biotin-label transfer assay. Labeling of putative surface receptors of caco-2 cells was performed using a biotin-labeled trifunctional cross-linking reagent as previously

described (Oliva *et al.*, 2008). Briefly, *C. difficile* spores (5 x 10^9 spores) were incubated with 1 ml 0.1 M bicarbonate buffer (pH 8.2)-1 mg of sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'dithiopropionate (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 in -80°C until use. Labeling reactions were carried in the dark. Next, the conjugated *C. difficile* spores were added at an MOI 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; Spectroline) at a distance of 6 cm for 20 min at 4°C. Cells were lysed in DPBS-0.1% Tween-0.4 % Triton X-100 in presence of protease inhibitors (Promega), the disulfide bond of the trifunctional cross-linker was reduced with 100 mM DTT, and Caco-2 cell biotin-labeled surface proteins were analyzed by Western blot using 1:10,000 streptavidin conjugated with IRDye 680 (Licor, Canada).

Statistical analyses. All experiments were carried out in duplicated and repeated at least 3 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, Inc).

RESULTS

***C. difficile* spores exhibit higher 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 higher 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 it 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 adherence, compared to spores of strain 630 (Fig. 1A). For comparison, we used spores of *C. perfrigens*, 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 (i.e., SM101 and F4969) had significantly lower levels of adherence to monolayers of Caco-2 cells than *C. difficile* spores (Fig. 1A).

To confirm the above results, spores of *C. difficile* 630 and *C. perfringens* F4969 were fluorescently labeled prior to infecting Caco-2 cells. Results indicate that *C. difficile* 630 spores adhered well to monolayers of Caco-2 cells and exhibited significantly ($p < 0.01$) higher adherence than fluorescently labeled *C. perfringens* F4969 spores (Fig. 1B). Collectively, these

results indicate that *C. difficile* spores exhibit higher adherence than *C. perfringens* spores to monolayers of Caco-2 cells.

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 labeled *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,B,C). Adherence of *C. difficile* 630 spores to untreated monolayers of Caco-2 cells was targeted to 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 adherence of *B. 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, *C. difficile* spores of strains (i.e., Pitt51 and Pitt177) with lower adherence to Caco-2 cells (Fig. 1A) had significantly ($p < 0.01$) lower spore surface hydrophobicity than spores of a strain (i.e., strain 630) with higher adherence to Caco-2 cells (Fig. 1A and 3), suggesting that hydrophobicity of the spore surface plays a role in adherence of *C. difficile* spores. However, despite the lower adherence observed with *C. perfringens* spores, these spores had similar levels of hydrophobicity than *C. difficile* strain 630 spores (Fig. 1A,B and 3). Strikingly, *B. subtilis* spores had significantly ($p < 0.01$) lower

hydrophobicity than spores of *C. difficile* and *C. perfringens* strains (Fig. 3). Collectively, these results suggest that i) *C. difficile* spores with higher hydrophobicity have higher adherence to Caco-2 cells; ii) *C. perfringens* spores have different surface proteins and/or different amounts of similar surface proteins found in *C. difficile* spores that promote adherence to Caco-2 cells; iii) *B. subtilis* adherence to Caco-2 cells does not require hydrophobic interactions.

Trypsin treatment reduces adherence of *C. difficile* spores to the apical surface of Caco-2 cells. In order to test the hypothesis that *C. difficile* spore-surface proteins are involved in adherence of *C. difficile* spores to monolayers of Caco-2 cells, *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 Caco-2 cells were infected with trypsin treated *C. difficile* spores, a ~ 4-fold reduction in adherence of viable spores was observed when 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 adherence of *C. difficile* spores 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 analyzed by scanning electron microscopy (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 on the ability of trypsin-treated *C. difficile* spores to adhere to Caco-2 cells, the fraction that did adhere did evenly to the surface of Caco-2 cells (Fig. 4C). Indeed, trypsin-treated spores that adhered to Caco-2 cells seemed to adhere through remnants of surface proteins involved in adherence to the microvilli similarly as untreated spores (Fig. 4B,D). Jointly, these results provide evidence that

340 *C. difficile* spores bind to enterocyte-like epithelial cells, and that spore-surface proteins are
341 involved in this adherence.
342
343 **Sonication alters the spores' ultrastructure by removing spore-surface proteins.** In *Bacillus*
344 *anthracis*, sonication has been reported to remove the exosporium (Kang *et al.*, 2005). Recently,
345 Lawley *et al*. (Lawley *et al.*, 2009) demonstrated that *C. difficile* spores have a premature
346 exosporium that lacks the hair-like nap typical of several *Bacillus* species. Indeed, our TEM of
347 negatively stained-*C. difficile* spores show the outer-most layer as a translucent layer with
348 absence of a hair-like nap structure (Fig. 5A). Osmium tetroxide-stained untreated *C. difficile*
349 630 spores show that surrounding the spore coats is a diffuse outer layer, likely the exosporium-
350 like structure, that differs significantly from the hair-like nap observed in several *Bacillus* species
351 (Henriques & Moran, 2007), confirming previous observations (Lawley *et al.*, 2009). This
352 exosporium-like structure surrounds another electron-dense layer (Fig. 5A), this being the spore
353 coats, with typical laminations that resemble the striated outer coats of *B. subtilis* spores
354 (Henriques & Moran, 2007). To evaluate if sonication would remove the exosporium-like
355 structure in *C. difficile* spores, *C. difficile* spores were sonicated and analyzed by TEM.
356 Interestingly, osmium tetroxide-stained sonicated spores lack the majority of the diffuse outer
357 layer (Fig 5A). Negatively-stained sonicated-spores lacked the outer-most diffuse translucent
358 layer (data not shown), indicating the majority of the diffuse layer surrounding the coats of *C.*
359 *difficile* spores can be removed by sonication.
360 To identify what proteins were removed by sonication, *C. difficile* spores' surface
361 proteins were biotinylated and subjected to either sonication or a decoating treatment as a control
362 of outer spore-protein layer removal. As expected, decoating treatment removed the majority of

363 the spore material surrounding the spore peptidoglycan cortex (Fig. 5B), presumably the coats
364 and the exosporium-like structure, with little biotinylated material remaining in decoated spores
365 as shown by Western blot analysis (data not shown). In contrast, sonication treatment only
366 released two protein species of ~ 40-45 kDa that were detectable in the supernatant fraction of
367 sonicated biotin-labeled spores by Western blot analysis (Fig. 5B). The highest and lower
368 molecular weight protein species correspond to ~ 72±8 and 23±8 % of the removed material,
369 respectively (Fig. 5B). This suggests that these two protein species might be the major
370 components of the outer-most diffuse layer or exosporium-like structure. It is likely that these
371 proteins were also present in coat extracts; however, their presence might be covered by more
372 abundant proteins of similar size (Fig. 5B). Interestingly, hydrophobicity of sonicated spores was
373 significantly lower than that of untreated spores (Fig. 5C), suggesting that the protein species of
374 ~ 40-45 kDa removed by sonication play a role in hydrophobicity of *C. difficile* 630 spores.
375 Collectively, these results suggest that the structural properties of the *C. difficile* spore
376 exosporium might be significantly different than that of *B. antrhacis*.
377
378 **Decoating and sonication reduces adherence of *C. difficile* spores to Caco-2 cells.** Since at
379 least some of the ~ 40-45 kDa protein species were removed in sonicated *C. difficile* 630 spores
380 and certainly all protein from decoated spores, we hypothesized that these sonicated and
381 decoated spores would have decreased adherence to monolayers of Caco-2 cells. Fluorescent
382 microscopy analyses demonstrated that a few, if any, decoated *C. difficile* 630 spores were able
383 to adhere to monolayers of Caco-2 cells (Fig. 6). Adherence of sonicated *C. difficile* 630 spores
384 to monolayers of Caco-2 cells was significantly reduced as compared to untreated spores (Fig.
385 6). Collectively, these results support the hypothesis that sonication removed some proteins (e.g.,

386 species of ~ 40-45 kDa) that have a role in adherence of *C. difficile* spores to monolayers of
387 Caco-2 cells.
388
389 **Pull down of putative receptor(s) of Caco-2 cells.** In an attempt to identify potential candidate
390 proteins in Caco-2 cells that might be recognized by *C. difficile* spores as spore-specific
391 receptor(s), we labeled *C. difficile* 630 spores with a heterobifunctional crosslinker, and infected
392 5 day-old monolayers of Caco-2 cells. Proteins directly interacting with *C. difficile* spores were
393 crosslinked by UV-light activation of the aryl azyl group, and the biotin group was transferred to
394 the unknown proteins by cleaving the disulfide bond. Biotinylated receptor(s) from spore free
395 lysate of biotinylated Caco-2 cells were pulled down with streptavidin beads and analyzed by
396 Western blot with streptavidin IRDye conjugate. Strikingly, four major protein species with
397 molecular weights ranging from ~ 26 to 40-kDa were detectable (Fig. 7). There was variation in
398 the relative abundance of these proteins as determined by densitometry analysis, with the most
399 abundant protein being the ~ 26 kDa band corresponding to ~ 48% of pulldown proteins. No
400 biotin-labeled protein was detected in Caco-2 cell extracts when infected with un-labeled *C.*
401 *difficile* spores (data not shown). Collectively, these results clearly suggest that *C. difficile* spores
402 are interacting with specific receptor(s) of Caco-2 cells.
403

DISCUSSIONS

The earliest event during CDI could be the adherence of *C. difficile* spores to the intestinal mucosa, which might 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 that this adherence would be mediated by one or more spore- and host-specific proteins. Since Caco-2 cells resemble small intestinal enterocyte-like cells, the implications of these findings to pathogenesis of *C. difficile* might have implications in increasing the adherence of ingested *C. difficile* spores to the small intestine, incrementing the reservoir of *C. difficile* spores throughout the digestive tract.

The spore surface of *Bacillus* species that possess an exosporium structure (i.e., *B. antrhacis* and *B. cereus*) is characterized by 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 structural features of this exosporium-like structure are significantly different than those of *B. anthracis* and *B. cereus* (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). Evidence for such differences comes from sonication of *C. difficile* spores. Sonication treatment typically stripes 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 outermost layer of *C. difficile* spores (i.e., spores lacked a diffuse-like outer most layer). It was most striking that sonicated spores had reduced hydrophobicity and lower levels of adherence than that of untreated *C. difficile* spores to Caco-2 cells. Collectively, these results suggest that the two ~40-45 kDa spore-specific proteins could be directly involved in adherence of *C. difficile* spores to Caco-2 cells and that the nature of such interaction might be hydrophobic. It is tempting to hypothesize that the lower adherence to monolayers of Caco-2 cells and lower hydrophobicity with 567 mM hexadecane of Pitt51 and Pitt177 spores as compared to 630 spores, could be attributed to lower abundance of both ~ 40-45 kDa protein species on the surface of Pitt51 and Pitt177 spores. Similar to the case of *B. subtilis* and *C. perfringens* spores, decoating treatment of *C. difficile* spores readily striped off of their outer layers, presumably the coats and exosporium-like layer. These decoated spores adhered little if any to Caco-2 cells and even to a lesser extent than sonicated spores, indicating that either sonication did not remove completely the ~ 40-45 kDa proteins involved in adherence and/or there are other spore-surface proteins that have auxiliary roles in adherence of *C. difficile* spores to Caco-2 cells.

Although adherence of *C. difficile* vegetative cells was not tested in this study, it has been previously reported to be ~ 1% of total cells adhered to Caco-2 cells (Dingle *et al.*, 2010; Dingle *et al.*, 2011), much lower than ~ 50 to 70% adherence of *C. difficile* spores, supporting 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 work although acceptable for *C. difficile* spores are not recommended for

C. difficile spore outgrowth studies, as germinated *C. difficile* spores are rapidly inactivated by 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 possesses specific proteins that promote attachment to the apical surface of Caco-2 cells, and in absence of these, binding becomes non-specific. In addition to the apical cells surface, a relevant site of adherence of *C. difficile* spores to the intestinal epithelial cells, presumably during the course of CDI, might be the cell periphery. 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 labs seeks to identify and characterize these putative *C. difficile* spore receptor(s).

C. difficile and *C. perfringens* are both anaerobic, spore-forming and enteric pathogens; however, the mechanism of pathogenesis of these bacteria is significantly different (Deneve *et al*., 2009; Paredes-Sabja & Sarker, 2009; Uzal & McClane, 2011). Indeed, the lower ability of *C. perfringens* spores, compared to *C. difficile* spores, to adhere to monolayers of Caco-2 cells indicates that, in contrast to *C. difficile*, adherence of *C. perfringens* spores to the intestinal epithelium is not a significant aspect of pathogenesis as might be the case in *C. difficile*. It is worth noting that although the spore-surface of both species has relatively similar level hydrophobicity, *C. perfringens* spores adhered less to Caco-2 cells. The opposite was the situation for *B. subtilis* spores, which although had lower spore surface hydrophobicity adhered to 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, the similar levels of adherence of spores of *B. subtilis* and *C. difficile* to Caco-2 cells 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 and 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 their hydrophobicity. Further studies will be required to identify the precise proteins in spores of *B. subtilis* and *C. perfringens* involved in these differences.

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Figure legends

Fig. 1.A,B. Adherence of *C. diffcile, C. perfringens* and *B. sutbilis* spores to human intestinal epithelial cells. A) Early (5 days) undifferentiated Caco-2 cells were infected at an MOI of 10 with spores of *C. difficile, C. perfringens* and *B. subtilis* (BS P832) strains and percentage of spore adherence was determined by measuring number of viable spore counts relative to initial spore counts as described in Experimental Procedures; B), Early (5 days) undifferentiated Caco-2 cells were infected at an MOI of 10 with fluorescently labeled *C. difficile* 630 and *C. perfringens* F4969 spores and the average number of spores counted in at least ten microscope fields with a magnification of 100X as described in Experimental Procedures. Data represents the average of at least three independent experiments and error bars are standard error from the mean. * indicate statistical difference ($p < 0.05$) between *B. subtilis* P832 and *C. difficile* strain 630. ** indicate statistical difference ($p < 0.01$) when strains Pitt51, Pitt177, F4969 and SM101 are compared to *C. difficile* strain 630

Fig. 2.A-C. Effect of EDTA on adherence of *C. difficile* spores to Caco-2 cells. A) Monolayers of 5 day old Caco-2 cells were pretreated with Ca^{2+}-free DPBS-0.1 mM EDTA prior to infection, chelation of Ca^{2+} with EDTA disrupts the intercellular junctions. EDTA treated Caco-2 cells were subsequently infected with fluorescently labeled *C. difficile* spores at an MOI of 4. The number of adhered fluorescently labeled *C. difficile* 630 spores was determined by fluorescent microscopy as described in Material and Methods. B, C) Representative fluorescent micrographs of untreated (B) and EDTA-treated (C) 5 day-old Caco-2 cells monolayers. Asterisks denote statistical difference ($p < 0.01$) between Control and EDTA treated cells.

Fig. 3. Comparison of hydrophobicity of *C. difficile, C. perfringens* and *B. subtilis* spores. Hydrophobicity of spores of *C. difficile* strains 630, Pitt51, Pitt177, *C. perfringens* strains F4969 and SM101, and *B. subtilis* strain PS832 was measured with 567 mM of hexadecane as described in Material and Methods. Data represents the average of at least three independent experiments and error bars are standard error from the mean. * indicate statistical difference ($p < 0.01$) when *B. subtilis* P832 was compared to *C. difficile* strain 630 or *C. perfringens* strains SM101 or F4969. ** indicate statistical difference ($p < 0.01$) when strains Pitt51, Pitt177, F4969 and SM101 are compared to *C. difficile* strain 630.

Fig. 4A,B,C,D. Scanning electron micrographs of adhered *C. difficile* spores to Caco-2 cells. Caco-2 cells monolayers of 5 day-old were infected at an MOI of 100 with untreated (A,B) and trypsin-treated (C,D) *C. difficile* 630 spores and analyzed through SEM as described in Material and Methods. The micrographs show that untreated and to a lesser extent trypsin-treated *C. difficile* spores interact with immature microvilli of apical surface of monolayers of Caco-2 cells highlighted by white arrows. Size bars are indicated by white bars and their size is indicated.

Fig. 5 A-D. Effect of decoating and sonication in the spore ultrastructure and spore proteins of *C. difficile*. A) Electron micrographs of negatively stained untreated (NS-Unt) or osmium tetroxide and uranyl acetate stained untreated (OT-Unt) and sonicated (OT-Son) *C. difficile* spores. Bar scale size is shown in each panel. Black arrows highlight exosporium-like structure, and the grey arrow highlights spore surface of sonicated spores lacking the exosporium-like structure. EX: exosporium-like structure; CT: coat; CX: spore peptidoglycan

cortex. B) *C. difficile* 630 spores were biotin-labeled and either decoated or sonicated as described in Material and Methods. Aliquots of coat/exosporium extracts (CE) and sonicated extracts (S) were separated by SDS-PAGE proteins transferred to a nitrocellulose membrane, and biotinylated proteins were detected with Streptavidin-IRDye 680 conjugate as described in Material and Methods. Arrows denote the only two detectable bands from material released by sonication treatment and blot is representative of two experiments. C) Effect of sonication on hydrophobicity of *C. difficile* spores. Hydrophobicity of untreated and sonicated *C. difficile* spores was measured with 567 mM of hexadecane as described in Material and Methods. Asterisks denote statistical significance at a $p < 0.01$.

Fig. 6. Adherence of decoated and sonicated *C. difficile* spores to Caco-2 monolayers. Caco-2 monolayers of 5 days-old were infected at an MOI of 10 with fluorescently labeled untreated, decoated, or sonicated *C. difficile* 630 spores and adhered spores counted by fluorescent microscopy. Asterisks note statistical difference ($p < 0.01$) with untreated spores.

Fig. 7. Pull down of putative biotin-labeled receptor proteins of Caco-2 cells. Western blot analysis of Caco-2 cells surface proteins interacting with *C. difficile* spores. Caco-2 cells were infected at an MOI of 50 for 1 h with Sulfo-SBED labeled *C. difficile* spores and cells were washed and UV-treated to induce transfer of the biotin-group from the spore to the surface proteins of Caco-2 cells. Spores were released from the protein-biotin-spore complex and biotinylated proteins were electrophoresed in 12% SDS-PAGE and Western blotted using streptavidin conjugated with IRDye 680 antibody as described in Material and Methods. Biotinylated proteins were detected with strepdavidin-IRDye 680 at a dilution of 1:15,000. Grey

arrows denote the pulldown biotinylated proteins and grey numbers are the mean of their relative abundance with a standard error < 2%.

Fig. 1

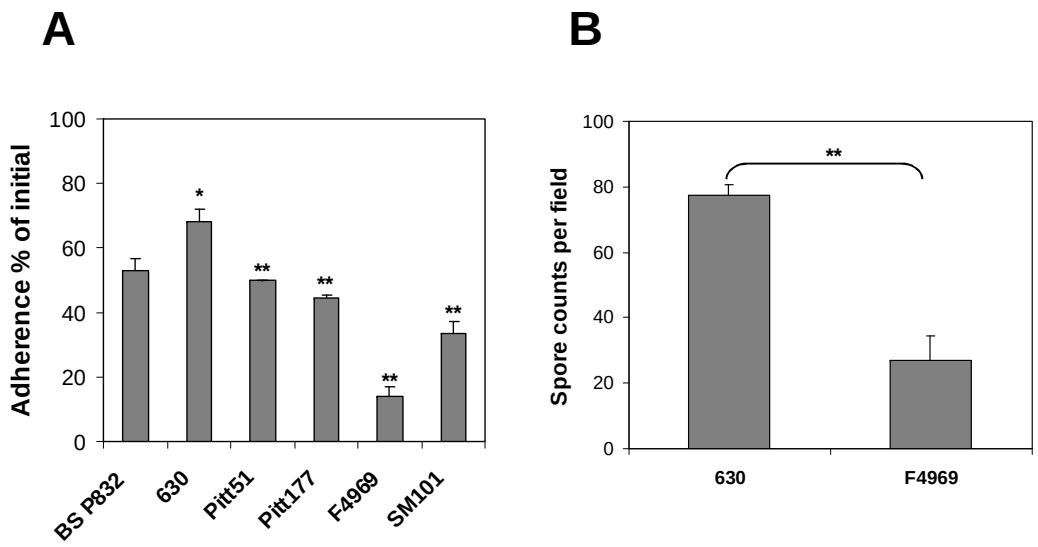
A
B
Adherence % of initial
100
80
60
40
20
0
*
**
**
**
**
BS P832
630
Pitt51
Pitt177
F4969
SM101
Spore counts per field
100
80
60
40
20
0
**
630
F4969

Fig. 2

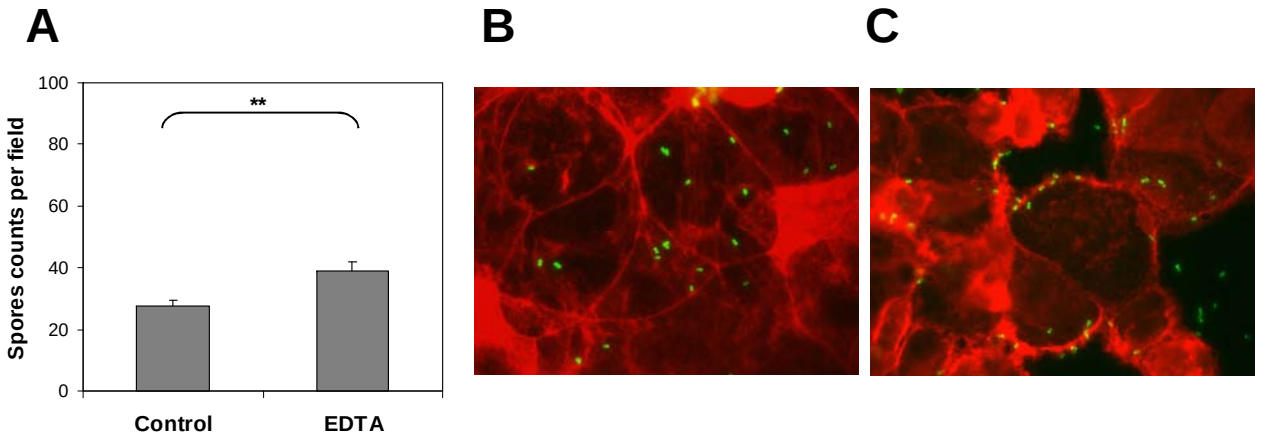

Fig. 3

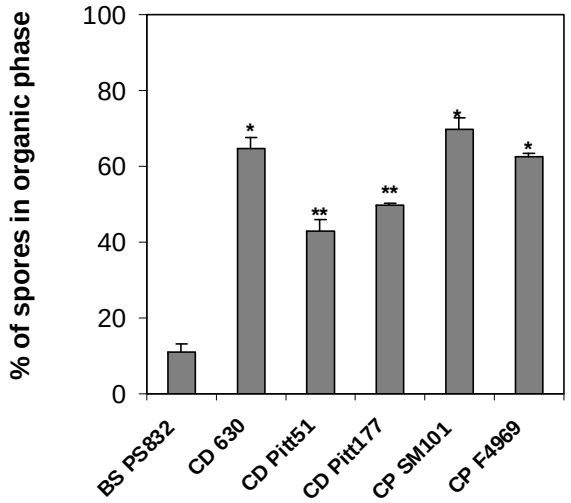
% of spores in organic phase
100
80
60
40
20
0
*
**
**
*
*
BS PS832
CD 630
CD Pitt51
CD Pitt177
CP SM101
CP F4969

Fig. 4

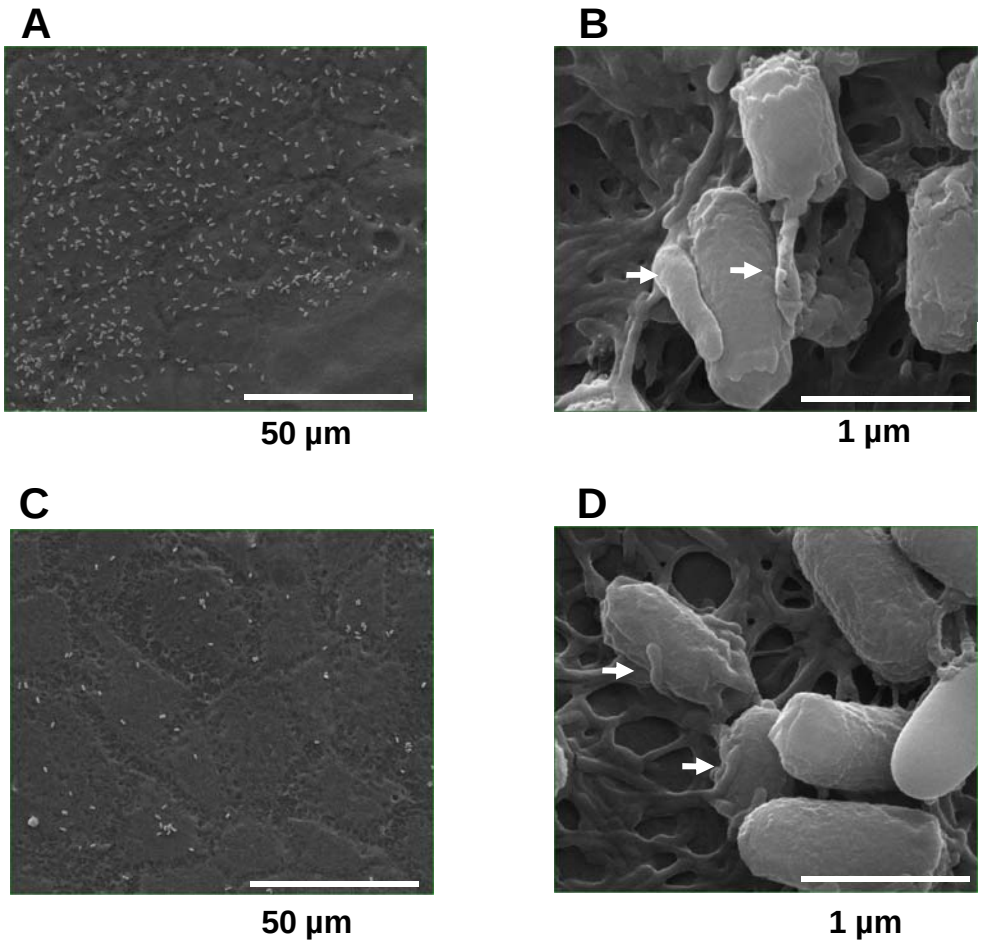

Fig. 5

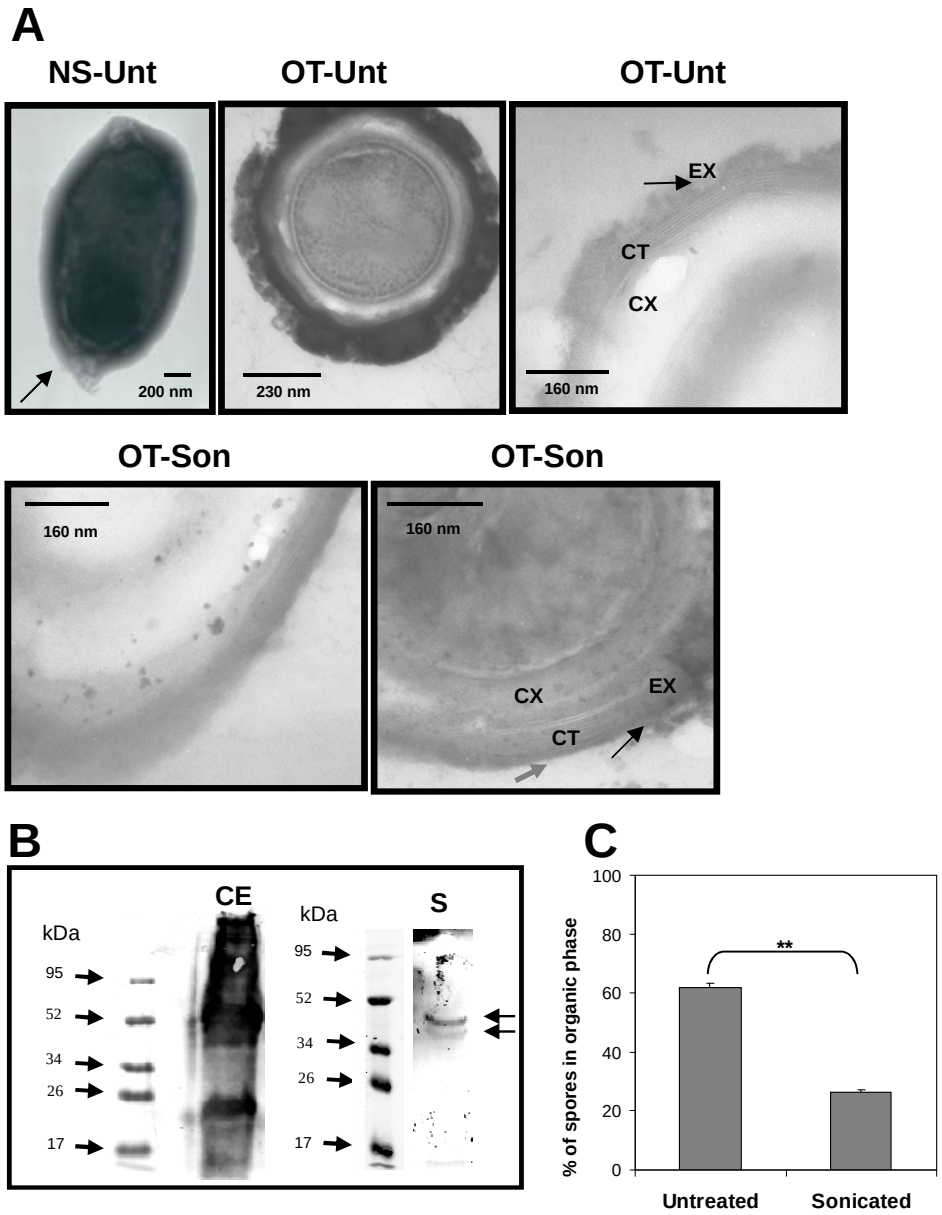
A
NS-Unt
OT-Unt
OT-Unt
200 nm
230 nm
EX
CT
CX
160 nm
OT-Son
OT-Son
160 nm
160 nm
CX
EX
CT
B
CE
S
kDa
95
52
34
26
17
kDa
95
52
34
26
17
C
% of spores in organic phase
100
80
60
40
20
0
**
Untreated
Sonicated

Fig. 6

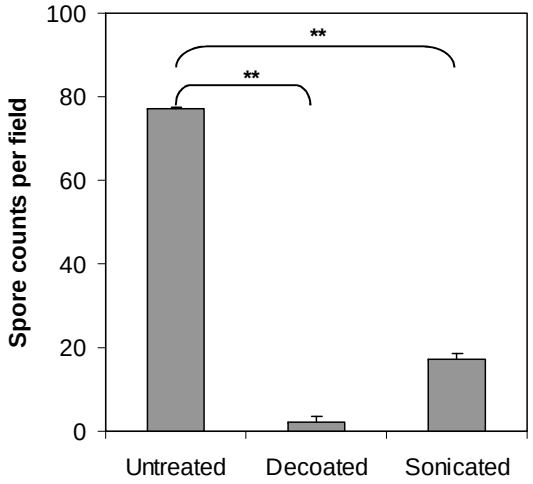
Spore counts per field
100
80
60
40
20
0
**
**
Untreated
Decoated
Sonicated

Fig. 7