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

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Abstract

22
23
24 *Clostridium difficile* is the causative agent of the majority of antibiotic associated diarrheas. *C.*
25 *difficile* spores are recognized as the morphotype of transmission, infection and persistence.
26 However, there is a lack of knowledge on how *C. difficile* spores interact with the host's
27 epithelial surfaces. In this context, we have characterized the ability of *C. difficile* spores to
28 adhere to human Caco-2 cells. Despite the similarities in spore-surface hydrophobicity between
29 spores of *C. difficile* and *C. perfringens* (another enteric pathogen that also sporulates in the gut),
30 spores of *C. difficile* adhere better to Caco-2 cells. Adherence to Caco-2 cells was significantly
31 reduced when *C. difficile* spores were trypsin-treated. Sonication of *C. difficile* spores altered the
32 ultrastructure of the outermost exosporium-like structure, released two protein species of ~ 40-
33 kDa and significantly reduced spore-hydrophobicity and adherence to Caco-2 cells. Using a
34 trifunctional crosslinker, we were able to co-immunoprecipitate four protein species from the
35 surface of Caco-2 cells. In conclusion, this study provides, for the first time evidence that *C.*
36 *difficile* spores adhere to human intestinal enterocyte-like cells through spore- and enterocytic-
37 surface specific ligand(s) and/or receptor(s).

38
39 Key Words: *C. difficile*, *C. perfringens*, Spores, Adherence.

40

41 INTRODUCTION

42

43 *Clostridium difficile* is a Gram-positive, anaerobic, spore-forming enteric pathogenic
44 bacterium, and the causative agent of pseudomembranous colitis and of 15 – 20% of antibiotic
45 associated diarrhea (Viswanathan *et al.*, 2010). *Clostridium difficile* infections (CDI) are a
46 significant cause of morbidity and economic losses of ~ 4 billion dollars annually in the United
47 States and European Union (Kuijper *et al.*, 2006). Antibiotic treatments disrupt the normal
48 intestinal microbiota, allowing the germinating *C. difficile* spore to outgrow and colonize the
49 host's intestinal tract, secreting toxins, and causing CDI. Two toxins, TcdA and TcdB, have been
50 identified as the major virulence factors in *C. difficile* pathogenesis (Kuehne *et al.*, 2010; Lyras
51 *et al.*, 2009; Voth & Ballard, 2005). They act as glycosyltransferases and modify small GTPases
52 of Rho protein family within the host cell, producing alterations in the cytoskeleton (Auwerx,
53 1991; Voth & Ballard, 2005). A third toxin, named CTD toxin is produced by few strains, also
54 plays a role in pathogenesis and increases adherence of *C. difficile* cells to host's epithelial
55 surfaces (Schwan *et al.*, 2009). However, the complexity of CDI symptoms suggests that other
56 less-studied non-toxin virulence factors and traits might be involved in *C. difficile* pathogenesis.

57 Although recent studies suggest that *C. difficile* epidemic strains do not have an
58 exceptionally high sporulation rate (Akerlund *et al.*, 2008; Merrigan *et al.*, 2010; Sirard *et al.*,
59 2011), there is a general consent that *C. difficile* spores are the persistent and infectious
60 morphotype as well as the vehicle of transmission of CDI. During the course of clinical
61 infections, an increase of *C. difficile* spores has been observed in the stools (Deneve *et al.*, 2009;
62 Hookman & Barkin, 2009; McFarland, 2005), indicating that *C. difficile* sporulates inside the
63 host leading to persistence of *C. difficile* spores in the intestinal tract. This is also supported by

64 high levels of sporulation of *C. difficile* observed in the intestinal tract of a hamster model
65 (Goulding *et al.*, 2009). The persistence of *C. difficile* spores in the colon of CDI-patients
66 complicates effective CDI treatments since *C. difficile* spores exhibit resistance to all currently
67 available treatments (McFarland, 2005) and can therefore survive in the colon until suppression
68 of CDI treatments. Interestingly, CDI has a relatively high rate (20%) of relapse (Pepin *et al.*,
69 2005), with ~ 25 to 85 % of the relapse being attributed to the initial strain (Barbut *et al.*, 2000;
70 O'Neill *et al.*, 1991; Oka *et al.*, 2012), suggesting that some strains of *C. difficile* might have
71 spores with unique properties to adhere to the host's intestinal epithelial surfaces.

72 *C. difficile* vegetative cells adhere to the specific components of the extracellular matrix
73 (ECM) such as fibrinogen, laminin, fibronectin, collagen I, III and IV (Cerquetti *et al.*, 2002).
74 Studies (Cerquetti *et al.*, 2002; Eveillard *et al.*, 1993) have shown that *C. difficile* vegetative cells
75 bind to intestinal epithelial cells, and interact with apical microvilli of differentiated Caco-2 cells
76 (Eveillard *et al.*, 1993). Several *C. difficile* cell surface proteins have been shown to play a role
77 in adherence to intestinal epithelial cells: i) a cell-surface protein (Cwp66) with adhesive
78 properties (Waligora *et al.*, 2001); ii) a fibronectin-binding protein, Fbp68 (Hennequin *et al.*,
79 2003); iii) S-layer proteins (Calabi *et al.*, 2002); and iv) the flagella, composed of the flagellin
80 FliC and the flagellar cap protein FliD, involved in mucus attachment (Tasteyre *et al.*, 2001).
81 However, to the best of our knowledge, there is a lack of information on the adherence of *C.*
82 *difficile* spores to intestinal epithelial cells. Therefore, in this study we have characterized the
83 adherence of *C. difficile* spores to cultured Caco-2 cells, an intestinal epithelial cell line
84 previously employed to characterize *in vitro* adherence of *C. difficile* vegetative cells to intestinal
85 epithelium (Drudy *et al.*, 2001; Eveillard *et al.*, 1993; Naaber *et al.*, 1996). This study, describes
86 the attachment of *C. difficile* spores to Caco-2 cells. Using biotinylation of Caco-2 cells' surface

87 proteins we were able to detect specific proteins of Caco-2 cells that interact with *C. difficile*
88 spores. Furthermore, we show that two specific *C. difficile* spore-proteins might be involved in
89 spore adherence.
90

91 **METHODS**

92

93 **Bacterial strains, human cell lines and chemicals.** *C. difficile* strains 630 (*tcdA*⁺, *tcdB*⁺, *tcdC*⁺,
94 *ctdA*⁻ and *ctdB*⁻), Pitt 51 (*tcdA*⁺, *tcdB*⁺, *tcdC*⁺, *ctdA*⁺ and *ctdB*⁺), Pitt 177 (*tcdA*⁺, *tcdB*⁺, *tcdC*⁺,
95 *ctdA*⁺ and *ctdB*⁺) (McEllistrem *et al.*, 2005; Paredes-Sabja & Sarker, 2011), *C. perfringens*
96 strains F4969 and SM101, and *Bacillus subtilis* strain PS832 are described elsewhere (Collie &
97 McClane, 1998; Paidhungat *et al.*, 2001; Waters *et al.*, 2003). *C. difficile* strains Pitt51 and
98 Pitt177 were isolated from patients presenting clinical symptoms of CDI in a tertiary care
99 hospital in Pittsburg, U.S.A. (McEllistrem *et al.*, 2005). Caco-2 cells were grown in eagle's
100 minimal essential medium (EMEM) (BioWhittaker, Lonza, Walkersville, MD) and used between
101 passages 50 and 80. Media was supplemented with 20% (v/v) fetal-calf serum (ATCC,
102 Manassas), penicillin (100 µg mL⁻¹) and streptomycin (100 µg mL⁻¹).

103

104 **Spore preparation and purification.** Preparation of *C. difficile* spores was done as described
105 (Paredes-Sabja & Sarker, 2011; Sorg & Sonenshein, 2008). Briefly, *C. difficile* strains were
106 grown in Brain Heart Infusion broth (Difco) supplemented with 0.5% yeast extract (Difco)
107 (BHIS) anaerobically at 37°C. Next overnight BHIS cultures were diluted to an OD₆₀₀ of 0.2 and
108 plated onto BHIS agar and incubated under anaerobic conditions at 37°C for 7 days (Paredes-
109 Sabja & Sarker, 2011; Sorg & Sonenshein, 2008). Sporulating cultures were recovered by
110 flooding the plates with ice-cold sterile distilled water, and collected by centrifugation. Cell
111 pellets were washed 10 times in ice cold water by repeated centrifugation and resuspension.
112 Next, free spores were separated using 50% HistoDenz and washed five times to eliminate traces

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

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

121 Spores of *Bacillus subtilis* were prepared by growing of 96 h at 37°C on BHI agar plates
122 as described (Nicholson & Setlow, 1990), and the spores were purified as described above.

123

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

132

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

136 Dulbecco's phosphate buffered saline (DPBS) (BioWhittaker, Lonza, Walkersville, MD), and
137 infected with *C. difficile* spores at a multiplicity of infection (MOI) of 4 or 10 in 200 μ l of
138 EMEM. Spore-infected Caco-2 cells were incubated for 1 h at 37°C under aerobic conditions.
139 Next, to remove unbound *C. difficile* spores from spore-infected Caco-2 cells, wells were washed
140 three times with DPBS and lysed with 100 μ l 0.06% Triton X-100 for 30 min at 37°C, plated
141 onto Brain Heart Infusion agar supplemented with 0.5% yeast extract, 2% glucose and 0.1%
142 sodium taurocholate (BHISG + ST) (Himedia Laboratories Pvt. Ltd. Mumbai, India), and
143 incubated under anaerobic conditions at 37°C overnight. For total *C. difficile* spores, spore-
144 infected Caco-2 cells wells were not washed and were directly lysed with 100 μ l 0.06% Triton
145 X-100 for 30 min at 37°C and lysed spore-infected Caco-2 cells directly plated onto BHISG +
146 ST agar plates, incubated anaerobically overnight at 37°C, and colony forming units (CFU) mL^{-1}
147 counted. Percentage of adherence was calculated using the following formula: [(Final CFU mL^{-1})
148 \div (Initial CFU mL^{-1})] x 100.

149 Adherence of *C. difficile* and *C. perfringens* spores was also quantified by fluorescent
150 microscopy. Briefly, Caco-2 cells were seeded (4×10^5 cells/well) onto 8-well culture slides (FD
151 Falcon) and incubated up to 5 days to a final density of 7×10^5 cells/well. Prior to infection,
152 confluent Caco-2 cells were washed three times with EMEM, and infected with 100 μ l of
153 EMEM containing Alexa Fluor 488-labeled *C. difficile* and *C. perfringens* spores at an MOI of 4
154 or 10 and incubated under aerobic conditions for 1 h at 37°C in a 5% CO_2 atmosphere.
155 Incubation up to 2 h did not significantly increase the ability of *C. difficile* spores to adhere to
156 Caco-2 cells (data not shown). Wells were washed three times with DPBS to remove unbound
157 labeled *C. difficile* spores, and fixed with 200 μ l of freshly prepared 4% paraformaldehyde for 15
158 min at room temperature and washed twice with DPBS. Next, cells were permeabilized with

159 0.06% Triton X-100 in DPBS for 15 min at 37°C, washed twice with DPBS and stained for F-
160 actin with 1 U of Alexa Fluor 568-phalloidin conjugate (Molecular Probes, Invitrogen, CA) for
161 30 min and rinsed three times with DPBS. To evaluate the effect of EDTA on adherence of *C.*
162 *difficile* spores to Caco-2 cells, 5-day old Caco-2 cell monolayers were pretreated with Ca²⁺-free
163 DPBS (Lonza)-0.1 mM EDTA tetrasodium salt (Versene, Lonza) for 1 h prior to infection in a
164 5% CO₂ atmosphere at 37°C, and subsequently infected at an MOI of 4 with *C. difficile* 630
165 spores in DPBS-0.1 mM EDTA tetrasodium salt (Versene, Lonza) for 1 h at 37°C in a 5% CO₂
166 atmosphere and treated as described above. Samples were air dried and mounted with Cytoseal
167 60 (Thermo Scientific) on cover slides and sealed with nail polish. DM4008B fluorescent
168 microscope was used to quantify total extra- and intra-cellular *C. difficile* and *C. perfringens*
169 spores adhered to Caco-2 cells. Photomicrographs were prepared with Adobe Photoshop and
170 Microsoft Picture Manager Software.

171
172 **Hydrophobicity assay.** Relative hydrophobicities of spores were measured by the bacterial
173 adherence to hydrocarbon (BATH) method (Brahmbhatt *et al.*, 2007; Rosenberg *et al.*, 1980).
174 Briefly, *C. difficile* and *C. perfringens* spores were re-suspended in sterile distilled water to a
175 final OD₄₄₀ ~ 0.5 and mixed with the non-aqueous solvent, hexadecane (Merck). Adherence to
176 hydrocarbons was measured by loss of turbidity in the aqueous phase. A ratio of 0.1 ml of
177 hydrocarbon mL⁻¹ of spore suspension yield sufficient separation (data not shown), which
178 corresponds to a 567 mM hexadecane. Suspensions were vortexed for 30 s, and phases allowed
179 to separate for 15 min at room temperature. Loss of turbidity of the aqueous solution was
180 measured and the hydrophobicity of spore's surface was calculated by the following formula:
181 $100 - [(Final\ OD_{440}) / (Initial\ OD_{440}) \times 100]$.

182

183 **Trypsin treatment of *C. difficile* spores.** *C. difficile* spores ($\sim 10^9$) were incubated with trypsin
184 (2.5 mg/ml) in 25 mM Phosphate buffered saline (pH 7.8) for 3 h at room temperature. Trypsin
185 treated *C. difficile* spores were washed five times with 150 mM NaCl and twice with distilled
186 water and stored in -20°C until use.

187

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

193 *C. difficile* spores were sonicated to remove the outermost surface layers as previously
194 described (Kang *et al.*, 2005). Briefly, *C. difficile* spores were re-suspended in 50 mM Tris-HCl,
195 0.5 mM EDTA buffer (pH 7.5). All subsequent manipulations were done in ice-cold conditions.
196 *C. difficile* spores were sonicated (Microson Ultrasonic Cell Disruptor XL, Misinox
197 Incorporated, Farmingdale, NY)) with maximum power (20 Watts) for 10 1-min burst, separated
198 by 3 min of cooling on ice. Sonicated spores were collected by centrifugation and washed three
199 times to eliminate loosely outermost surface layers. Sonicated spores were quantified with a
200 Heber Bacteria Counting Chamber Z300 (Hawksley, UK) and stored at -20°C until use.
201 Sonication had no effect on viability of *C. difficile* spores (data not shown).

202 In other experiments, *C. difficile* spores (4×10^9 spores) were labeled with 1 ml of 0.1 M
203 Bicarbonate buffer (pH 8.2) containing 2 mg of Biotinamido Hexanoic Acid N-
204 Hydroxysuccinimide 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

228 cacodylate buffer (pH 7.2), rinsed in cacodylate buffer, stained for 30 min with 1% tannic acid,
229 and rinsed off with cacodylate buffer. Samples were dehydrated with a step-wise acetone
230 gradient of 30% (stained with 2% uranyl acetate at this stage) for 30 min, 50% for 30 min, 70%
231 overnight, 90% for 30 min, and twice with 100% acetone. Dehydrated samples were embedded
232 in spurs at a ratio of acetone:spurs of 3:1, 1:1 and 1:3 for 40 min each, resuspended in 100%
233 spurs resin for 4 h and baked overnight at 65°C. Thin sections were obtained using a micotomb
234 and were placed on a glow discharge carbon-coated grids for negative staining and double lead
235 stained with 2% uranyl acetate and lead citrate. Samples were evaluated at 80 kV with a Philips
236 EM300 TEM at the Electron Microscopy Facility at Oregon State University and with a Phillips
237 Tecnai 12 Bio Twin at the Electron Microscopy facility at Pontificia Universidad Catolica de
238 Chile.

239
240 **Syto Green Fluorescence labeling of decoated *C. difficile* spores.** Since decoated *C. difficile*
241 spores were inefficiently labeled with Alexa Fluor 488, primarily because of primary amine
242 groups required by the TFP esters present in Alexa dyes for labeling, might have been efficiently
243 removed during decoating treatment. Therefore, decoated *C. difficile* spores were labeled in
244 phosphate buffered saline (PBS)-10 µM Syto Green Fluorescent Nucleic Acid Stain (Molecular
245 Probes, Invitrogen, U.S.A.) for 3 h at 4°C and labeling efficiency was evaluated by fluorescence
246 microscopy. Syto-labeled spores were counted with a Heber Bacteria Counting Chamber Z300
247 (Hawksley, UK) and stored at -20°C until use.

248
249 **Photo cross-linking biotin-label transfer assay.** Labeling of putative surface receptors of caco-
250 2 cells was performed using a biotin-labeled trifunctional cross-linking reagent as previously

251 described (Oliva *et al.*, 2008). Briefly, *C. difficile* spores (5×10^9 spores) were incubated with 1
252 ml 0.1 M bicarbonate buffer (pH 8.2)-1 mg of sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-
253 2-(p-azido benzamido)-hexanoamido) ethyl-1,3'dithiopropionate (Sulfo-SBED) (Pierce) for 30
254 min at room temperature. Conjugated *C. difficile* spores were washed three times with DPBS,
255 quantified as described above and stored in -80°C until use. Labeling reactions were carried in
256 the dark. Next, the conjugated *C. difficile* spores were added at an MOI of 50 to cells in petri
257 dishes and incubated at 37°C for 1 h in the dark. Infected Caco-2 cells were washed twice to
258 eliminate unbound conjugated spores, and subsequently treated with UV light at 365 nm (XX-
259 15B lamp; Spectroline) at a distance of 6 cm for 20 min at 4°C . Cells were lysed in DPBS-0.1%
260 Tween-0.4 % Triton X-100 in presence of protease inhibitors (Promega), the disulfide bond of
261 the trifunctional cross-linker was reduced with 100 mM DTT, and Caco-2 cell biotin-labeled
262 surface proteins were analyzed by Western blot using 1:10,000 streptavidin conjugated with
263 IRDye 680 (Licor, Canada).

264

265 **Statistical analyses.** All experiments were carried out in duplicated and repeated at least 3 times.
266 In some experiments Student's *t* test was used to compare specific treatments, and $P < 0.05$ was
267 considered significant using the statistical software Statgraphics Centurion XVI (StatPoint
268 Technologies, Inc).

269

270

271

272 **RESULTS**

273

274 ***C. difficile* spores exhibit higher adherence to cultured Caco-2 cells than *C. perfringens* and**

275 ***B. subtilis* spores.** Preliminary experiments of adherence of *C. difficile* 630 spores under aerobic

276 and anaerobic conditions gave essentially the same results (data not shown). Therefore all

277 subsequent experiments were conducted under aerobic conditions. First, we evaluated if *C.*

278 *difficile* spores had higher ability to adhere to Caco-2 cells than spores of other spore-forming

279 species. As a negative control, we used spores of *B. subtilis*, as it does not colonize the intestinal

280 tract. We incubated *B. subtilis* P832 spores with Caco-2 cells under aerobic conditions and the

281 fraction of adhered spores was quantified by viable counts. Unexpectedly nearly 50% of *B.*

282 *subtilis* spores adhered to monolayers of Caco-2 cells (Fig. 1A)., while nearly 70% of *C. difficile*

283 spores of strain 630 adhered to monolayers of Caco-2 cells (Fig. 1A). Spores of *C. difficile*

284 strains Pitt51 and Pitt177 exhibited slightly lower adherence, compared to spores of strain 630

285 (Fig. 1A). For comparison, we used spores of *C. perfringens*, another anaerobic enteric pathogen

286 whose spores lack the exosporium-like structure (Novak *et al.*, 2003; Orsburn *et al.*, 2008). *C.*

287 *perfringens* spores of two gastrointestinal disease related isolates (i.e., SM101 and F4969) had

288 significantly lower levels of adherence to monolayers of Caco-2 cells than *C. difficile* spores

289 (Fig. 1A).

290 To confirm the above results, spores of *C. difficile* 630 and *C. perfringens* F4969 were

291 fluorescently labeled prior to infecting Caco-2 cells. Results indicate that *C. difficile* 630 spores

292 adhered well to monolayers of Caco-2 cells and exhibited significantly ($p < 0.01$) higher

293 adherence than fluorescently labeled *C. perfringens* F4969 spores (Fig. 1B). Collectively, these

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

296 To evaluate if *C. difficile* spores also adhere better to the cell periphery of Caco-2 cells,
297 monolayers of Caco-2 cells were pretreated with the Ca²⁺-chelating agent (EDTA), and
298 subsequently infected with fluorescently labeled *C. difficile* 630 spores. Notably, *C. difficile*
299 spores adhered significantly ($p < 0.01$) better to EDTA-treated than to untreated monolayers of
300 Caco-2 cells (Fig. 2A,B,C). Adherence of *C. difficile* 630 spores to untreated monolayers of
301 Caco-2 cells was targeted to whole apical surface of Caco-2 cells (Fig. 2B), whereas *C. difficile*
302 spores mainly adhered to the periphery of EDTA-treated Caco-2 cells (Fig. 2C). These results
303 indicate that *C. difficile* spores adhere better to the periphery than to the apical surface of 5 day
304 old monolayers of EDTA-treated Caco-2 cells.

305

306 **Spore surface hydrophobicity is involved in adherence of *C. difficile* but not *C. perfringens***
307 **spores.** Hydrophobic interactions have been shown to be involved in adherence of *B. cereus*
308 spores to Caco-2 cells (Andersson *et al.*, 1998). Therefore, to evaluate whether the observed
309 adherence would correlate with spore surface hydrophobicity we measured the percentage of
310 removed spores with 567 mM hexadecane. Interestingly, *C. difficile* spores of strains (i.e., Pitt51
311 and Pitt177) with lower adherence to Caco-2 cells (Fig. 1A) had significantly ($p < 0.01$) lower
312 spore surface hydrophobicity than spores of a strain (i.e., strain 630) with higher adherence to
313 Caco-2 cells (Fig. 1A and 3), suggesting that hydrophobicity of the spore surface plays a role in
314 adherence of *C. difficile* spores. However, despite the lower adherence observed with *C.*
315 *perfringens* spores, these spores had similar levels of hydrophobicity than *C. difficile* strain 630
316 spores (Fig. 1A,B and 3). Strikingly, *B. subtilis* spores had significantly ($p < 0.01$) lower

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

322

323 **Trypsin treatment reduces adherence of *C. difficile* spores to the apical surface of Caco-2**
324 **cells.** In order to test the hypothesis that *C. difficile* spore-surface proteins are involved in
325 adherence of *C. difficile* spores to monolayers of Caco-2 cells, *C. difficile* 630 spores were
326 trypsinized and assayed for adherence. No reduction in spore viability was observed in trypsin
327 treated spores (data not shown). Interestingly, when Caco-2 cells were infected with trypsin
328 treated *C. difficile* spores, a ~ 4-fold reduction in adherence of viable spores was observed when
329 compared to untreated *C. difficile* spores (data not shown), suggesting that *C. difficile* spore
330 surface proteins, which are removed at least in part by trypsin treatment, are involved in
331 adherence to Caco-2 cells. To gain more insight into the topology of adherence of *C. difficile*
332 spores to enterocyte-like epithelial cells, Caco-2 cells were infected with untreated (Fig. 4A,B)
333 and trypsin-treated (Fig. 4C,D) *C. difficile* 630 spores and analyzed by scanning electron
334 microscopy (SEM). Interestingly, SEM showed that *C. difficile* spores adhered evenly to the
335 surface of Caco-2 cells (Fig. 4A). Although there was a significant reduction on the ability of
336 trypsin-treated *C. difficile* spores to adhere to Caco-2 cells, the fraction that did adhere did
337 evenly to the surface of Caco-2 cells (Fig. 4C). Indeed, trypsin-treated spores that adhered to
338 Caco-2 cells seemed to adhere through remnants of surface proteins involved in adherence to the
339 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. anthracis*.

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

404 **DISCUSSIONS**

405

406 The earliest event during CDI could be the adherence of *C. difficile* spores to the
407 intestinal mucosa, which might play a significant role in persistence of *C. difficile* spores in
408 healthy individuals, as well as in CDI relapse episodes. In this context, our results provide
409 evidence that *C. difficile* spores are able to adhere to enterocyte-like epithelial cells and that this
410 adherence would be mediated by one or more spore- and host-specific proteins. Since Caco-2
411 cells resemble small intestinal enterocyte-like cells, the implications of these findings to
412 pathogenesis of *C. difficile* might have implications in increasing the adherence of ingested *C.*
413 *difficile* spores to the small intestine, incrementing the reservoir of *C. difficile* spores throughout
414 the digestive tract.

415 The spore surface of *Bacillus* species that possess an exosporium structure (i.e., *B.*
416 *anthracis* and *B. cereus*) is characterized by the presence of a hair-like nap that surrounds the
417 basal layer of the exosporium (Henriques & Moran, 2007). Although it is suggested that *C.*
418 *difficile* spores possess an exosporium-like structure (Lawley *et al.*, 2009), the structural features
419 of this exosporium-like structure are significantly different than those of *B. anthracis* and *B.*
420 *cereus* (Henriques & Moran, 2007). In this context, our TEM results are in agreement with
421 previous findings (Lawley *et al.*, 2009), and highlight the absence of a hair-like nap, suggesting
422 that the diffuse-layer that surrounds *C. difficile* spores might be structurally and functionally
423 different than that of *B. anthracis* and *B. cereus* (Henriques & Moran, 2007). Evidence for such
424 differences comes from sonication of *C. difficile* spores. Sonication treatment typically stripes off
425 the exosporium fraction in *B. anthracis* spores which is composed of ~ 20 proteins (Henriques &
426 Moran, 2007), however, only two *C. difficile* proteins of ~40-45 kDa were efficiently extracted

427 when biotinylated *C. difficile* spores were subjected to sonication. Absence of these two protein
428 species significantly affected the ultrastructure of the exosporium-like outermost layer of *C.*
429 *difficile* spores (i.e., spores lacked a diffuse-like outer most layer). It was most striking that
430 sonicated spores had reduced hydrophobicity and lower levels of adherence than that of
431 untreated *C. difficile* spores to Caco-2 cells. Collectively, these results suggest that the two ~40-
432 45 kDa spore-specific proteins could be directly involved in adherence of *C. difficile* spores to
433 Caco-2 cells and that the nature of such interaction might be hydrophobic. It is tempting to
434 hypothesize that the lower adherence to monolayers of Caco-2 cells and lower hydrophobicity
435 with 567 mM hexadecane of Pitt51 and Pitt177 spores as compared to 630 spores, could be
436 attributed to lower abundance of both ~ 40-45 kDa protein species on the surface of Pitt51 and
437 Pitt177 spores. Similar to the case of *B. subtilis* and *C. perfringens* spores, decoating treatment of
438 *C. difficile* spores readily striped off of their outer layers, presumably the coats and exosporium-
439 like layer. These decoated spores adhered little if any to Caco-2 cells and even to a lesser extent
440 than sonicated spores, indicating that either sonication did not remove completely the ~ 40-45
441 kDa proteins involved in adherence and/or there are other spore-surface proteins that have
442 auxiliary roles in adherence of *C. difficile* spores to Caco-2 cells.

443 Although adherence of *C. difficile* vegetative cells was not tested in this study, it has
444 been previously reported to be ~ 1% of total cells adhered to Caco-2 cells (Dingle *et al.*, 2010;
445 Dingle *et al.*, 2011), much lower than ~ 50 to 70% adherence of *C. difficile* spores, supporting
446 the hypothesis that spore adherence might be exploited by *C. difficile* as a means of persistence
447 in the host. Despite the fact that the colonic environment is mostly anaerobic, there is an oxygen
448 concentration gradient from the colonic lumen to the colonic epithelium, the experimental
449 conditions used in this work although acceptable for *C. difficile* spores are not recommended for

450 *C. difficile* spore outgrowth studies, as germinated *C. difficile* spores are rapidly inactivated by
451 presence of oxygen. Trypsin treatment of *C. difficile* spores showed that adherence was
452 dependent on spore-surface proteins that were cleaved by trypsin, suggesting that *C. difficile*
453 spores also possesses specific proteins that promote attachment to the apical surface of Caco-2
454 cells, and in absence of these, binding becomes non-specific. In addition to the apical cells
455 surface, a relevant site of adherence of *C. difficile* spores to the intestinal epithelial cells,
456 presumably during the course of CDI, might be the cell periphery. However, the increase of
457 adherence to EDTA-treated cells, although significant was small and might not hold biological
458 relevance. Co-immunoprecipitation experiments demonstrated that *C. difficile* spores interact
459 specifically with proteins on the surface of apical microvilli of Caco-2 cells, strongly supporting
460 the hypothesis that *C. difficile* spores adhere to specific receptor protein(s) on the surface of the
461 apical microvilli of Caco-2 cells. Work in our labs seeks to identify and characterize these
462 putative *C. difficile* spore receptor(s).

463 *C. difficile* and *C. perfringens* are both anaerobic, spore-forming and enteric pathogens;
464 however, the mechanism of pathogenesis of these bacteria is significantly different (Deneve *et*
465 *al.*, 2009; Paredes-Sabja & Sarker, 2009; Uzal & McClane, 2011). Indeed, the lower ability of *C.*
466 *perfringens* spores, compared to *C. difficile* spores, to adhere to monolayers of Caco-2 cells
467 indicates that, in contrast to *C. difficile*, adherence of *C. perfringens* spores to the intestinal
468 epithelium is not a significant aspect of pathogenesis as might be the case in *C. difficile*. It is
469 worth noting that although the spore-surface of both species has relatively similar level
470 hydrophobicity, *C. perfringens* spores adhered less to Caco-2 cells. The opposite was the
471 situation for *B. subtilis* spores, which although had lower spore surface hydrophobicity adhered
472 to similar levels as *C. difficile* spores. *B. subtilis* has been shown to be able to sporulate in the

473 intestinal tract but it does not colonize the intestinal tract (Tam *et al.*, 2006). However, the
474 similar levels of adherence of spores of *B. subtilis* and *C. difficile* to Caco-2 cells suggest that
475 either *B. subtilis* spores are indeed able to adhere to the intestinal epithelium or that the
476 adherence assay might not be a direct indicator of the ability of spores to adhere to the intestinal
477 epithelium and this experimental assay might require further improvement. Collectively, these
478 results highlight significant differences in the spore surface of these three species, and the
479 differential nature of the interactions that promote adherence, where at least for *C. difficile*
480 spores, adherence is modulated by the presence of two protein species of 40-45 kDa that
481 contribute to their hydrophobicity. Further studies will be required to identify the precise
482 proteins in spores of *B. subtilis* and *C. perfringens* involved in these differences.

483

484

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Figure legends

688

689 **Fig. 1.A,B. Adherence of *C. difficile*, *C. perfringens* and *B. subtilis* spores to human intestinal**

690 **epithelial cells.** A) Early (5 days) undifferentiated Caco-2 cells were infected at an MOI of 10

691 with spores of *C. difficile*, *C. perfringens* and *B. subtilis* (BS P832) strains and percentage of

692 spore adherence was determined by measuring number of viable spore counts relative to initial

693 spore counts as described in Experimental Procedures; B), Early (5 days) undifferentiated Caco-

694 2 cells were infected at an MOI of 10 with fluorescently labeled *C. difficile* 630 and *C.*

695 *perfringens* F4969 spores and the average number of spores counted in at least ten microscope

696 fields with a magnification of 100X as described in Experimental Procedures. Data represents the

697 average of at least three independent experiments and error bars are standard error from the

698 mean. * indicate statistical difference ($p < 0.05$) between *B. subtilis* P832 and *C. difficile* strain

699 630. ** indicate statistical difference ($p < 0.01$) when strains Pitt51, Pitt177, F4969 and SM101

700 are compared to *C. difficile* strain 630

701

702 **Fig. 2.A-C. Effect of EDTA on adherence of *C. difficile* spores to Caco-2 cells.** A)

703 Monolayers of 5 day old Caco-2 cells were pretreated with Ca^{2+} -free DPBS-0.1 mM EDTA prior

704 to infection, chelation of Ca^{2+} with EDTA disrupts the intercellular junctions. EDTA treated

705 Caco-2 cells were subsequently infected with fluorescently labeled *C. difficile* spores at an MOI

706 of 4. The number of adhered fluorescently labeled *C. difficile* 630 spores was determined by

707 fluorescent microscopy as described in Material and Methods. B, C) Representative fluorescent

708 micrographs of untreated (B) and EDTA-treated (C) 5 day-old Caco-2 cells monolayers.

709 Asterisks denote statistical difference ($p < 0.01$) between Control and EDTA treated cells.

710

711 **Fig. 3. Comparison of hydrophobicity of *C. difficile*, *C. perfringens* and *B. subtilis* spores.**

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

719

720 **Fig. 4A,B,C,D. Scanning electron micrographs of adhered *C. difficile* spores to Caco-2 cells.**

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

726

727 **Fig. 5 A-D. Effect of decoating and sonication in the spore ultrastructure and spore**

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

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

742

743 **Fig. 6. Adherence of decoated and sonicated *C. difficile* spores to Caco-2 monolayers.** Caco-
744 2 monolayers of 5 days-old were infected at an MOI of 10 with fluorescently labeled untreated,
745 decoated, or sonicated *C. difficile* 630 spores and adhered spores counted by fluorescent
746 microscopy. Asterisks note statistical difference ($p < 0.01$) with untreated spores.

747

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

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

Fig. 1

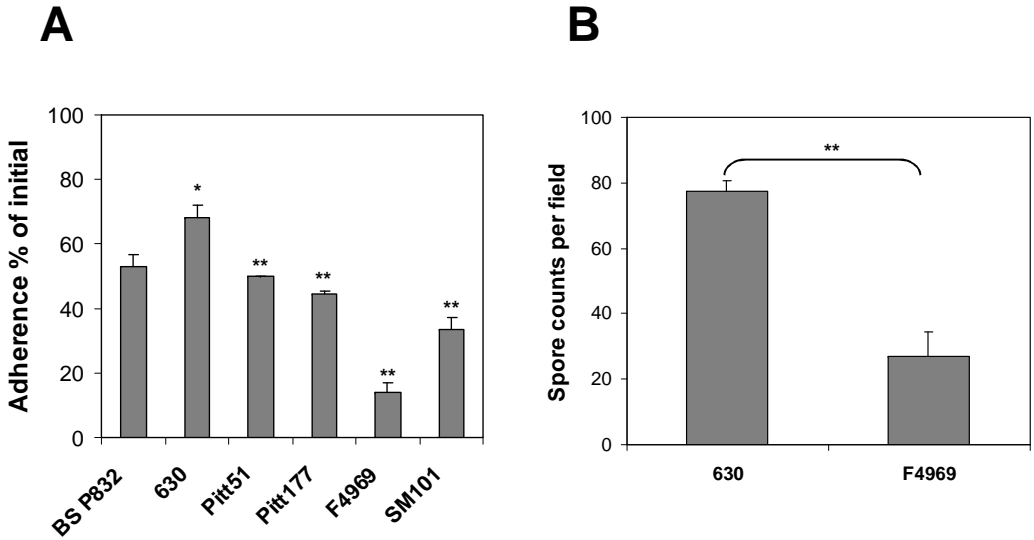


Fig. 2

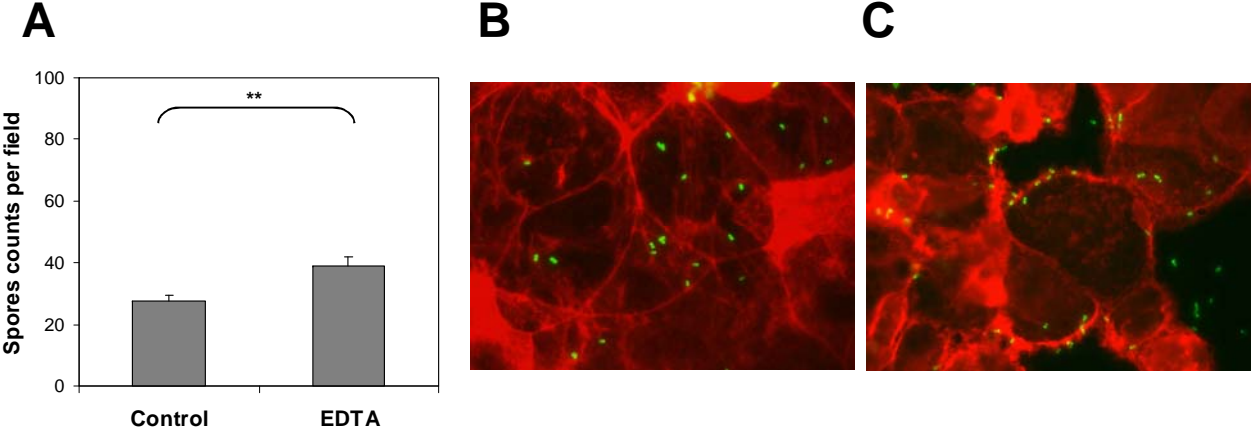


Fig. 3

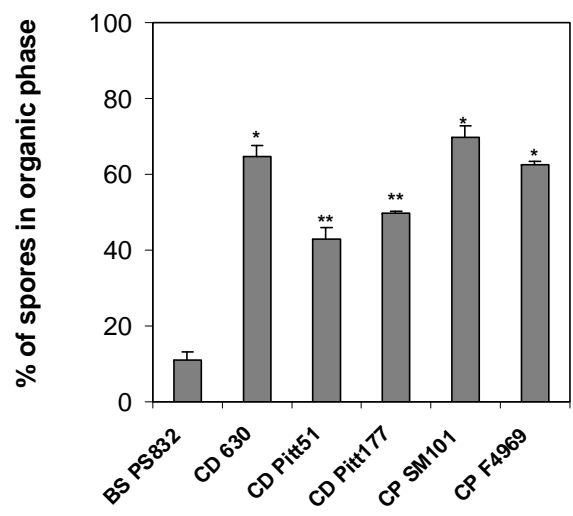
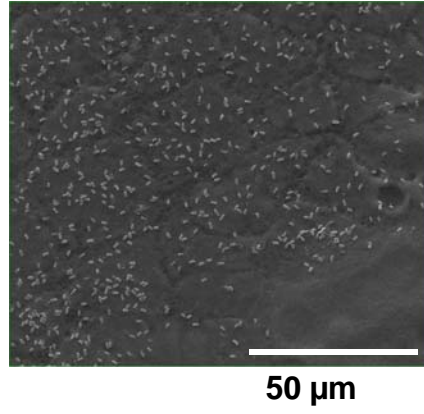
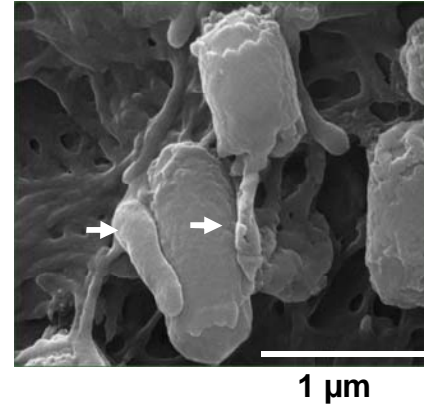


Fig. 4

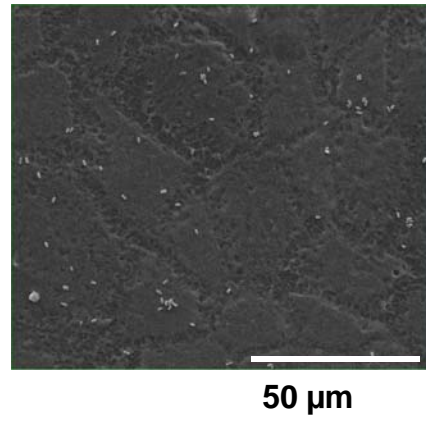
A



B



C



D

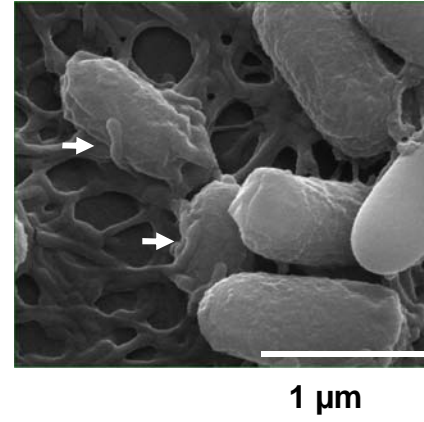


Fig. 5

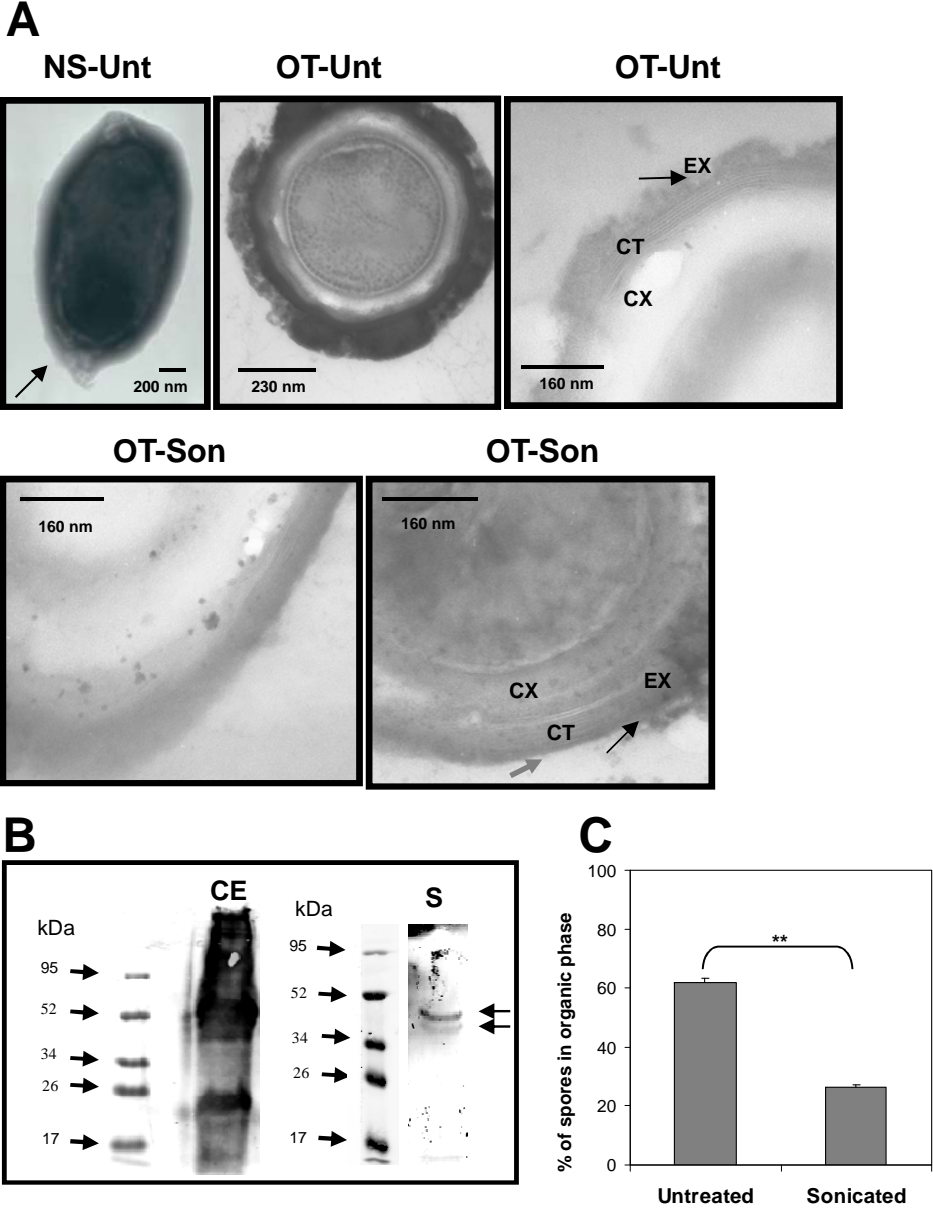


Fig. 6

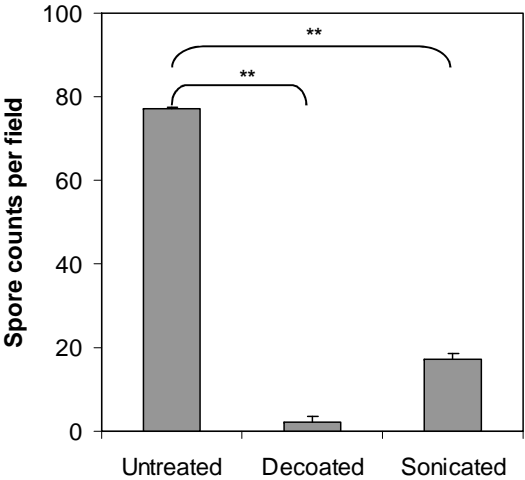


Fig. 7

