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1 **Surface Morphology Differences in *Clostridium difficile* Spores, Based**
2 **on Different Strains and Methods of Purification**

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11 **Abstract**

12 Infections linked to *Clostridium difficile* are a significant cause of suffering. In hospitals, the
13 organism is primarily acquired through the faecal-oral route as spores excreted by infected patients
14 contaminate the healthcare environment. We previously reported that members of the *C. difficile*
15 group varied widely in their ability to adhere to stainless steel and proposed that these differences
16 were a consequence of variations in spore architecture. In this study of clinical isolates and spore
17 coat protein mutants of *C. difficile* we identified three distinct spore surfaces morphotypes; smooth,
18 bag-like and “pineapple-like” using scanning electron microscopy (SEM). The frequency of each
19 morphotype in a spore population derived from a single isolate varied depending on the host strain
20 and the method used to produce and purify the spores. Our results suggest that the inclusion of a
21 sonication step in the purification process had a marked effect on spore structure. In an attempt to
22 link differences in spore appearance with key structural spore proteins we compared the morphology
23 of spores of CD630 to those produced by CD630 variants lacking either CotE or BclA. While SEM
24 images revealed no obvious structural differences between CD630 and its mutants we did observe
25 significant differences ($p < 0.001$) in relative hydrophobicity suggesting that modifications had
26 occurred but not at a level to be detectable by SEM.

27 In conclusion, we observed significant variation in the spore morphology of clinical isolates of *C.*
28 *difficile* due in part to the methods used to produce them. Sonication in particular can markedly
29 change spore appearance and properties. The results of this study highlight the importance of
30 adopting “standard” methods when attempting to compare results between studies and to understand
31 the significance of their differences.

32 1. Introduction

33 *Clostridium difficile* (recently renamed as *Clostridioides difficile*) is a Gram-positive anaerobic
34 spore-forming bacterium and is currently the most common cause of antibiotic-associated
35 nosocomial infection the US and UK [1, 2]. It is estimated that in 2015 the pathogen was responsible
36 for 500,000 cases and 15,000 deaths in the US, according to the CDC [3]. The most recent data from
37 the UK reported 12,798 confirmed cases in England in 2016-2017 [4]. While the incidence in the
38 UK has reduced significantly from a peak of 60,000 cases in 2007, it still remains a significant cause
39 of suffering. The additional costs associated with treating infected individuals, estimated to be
40 between £4,000 (\$5,500) and £8,000 (\$11,000) impose a significant financial burden on healthcare
41 systems [5]. Alterations in colonic microbiota, usually due to broad-spectrum antibiotic treatment,
42 increase sensitivity to *C. difficile* infection and enable the vegetative organism to produce cytotoxins
43 that destroy host intestinal epithelial cells [6].

44 In the hospital environment the organism is primarily acquired through the faecal-oral route
45 contaminating the surrounding healthcare environment [7, 8]. Current measures to prevent the
46 spread of the pathogen seek to block transmission routes and to limit inappropriate antibiotic usage
47 [9]. The ability of spores to adhere to surfaces is thought to play an important role in their survival
48 and spread and in the subsequent infection of susceptible individuals [9, 10]. Adherence to organic
49 and inorganic surfaces is influenced by a number of factors, which include hydrophobicity and the
50 presence of surface structures, such as appendages [11] and the outer spore layer known as the
51 exosporium [12-15].

52 *C. difficile* shows significant variation within the species. There are over 100 existing ribotypes with
53 each ribotype being a broad genetic group based on rRNA similarities [16]. Some ribotypes, such
54 as the 027 ribotype are associated with higher virulence, and have been subject of study. In an earlier
55 study we showed that clinical isolates of *C. difficile* varied widely in their inherent hydrophobicity
56 and in their ability to adhere to stainless steel. These differences which were independent of ribotype,
57 appeared to be linked to the presence of an exosporium-like layer [17]. Further characterisation of
58 the spore morphotypes and particularly the exosporium layer was necessary to understand the
59 variation both between different ribotypes and within the same ribotype, which became the focus of
60 this research.

61 The exosporium is a loose outer layer, which surrounds the spores produced by some, but not all,
62 *Bacillus* and *Clostridium* species, [12, 13, 18-20]. In the case of *Bacillus cereus* and *anthracis*
63 species it forms a loose “baggy” layer surrounding the spore with hair-like appendages projecting
64 from the surface [21]. Studies have reported that the outer spore layer of clinical isolates of *C.*
65 *difficile* differ markedly [22]. For some isolates the exosporium appears to be tightly bound to the
66 spore coat, which in others forms a bag-like structure [14, 22-24]. Several proteins were found to be
67 essential for *C. difficile* spore outer layer assembly, including the *BclA*, *Cot* and *CdeC* families of
68 proteins [25]. To add to our understanding of these proteins, we looked contribution of individual
69 known spore structural proteins to spore surface morphology.

70 The analysis of spore surface structure is further complicated by the fact that there is no common
71 spore production method making it difficult to compare the results between studies. To ensure that
72 the results are relevant to the real-world properties of *C. difficile* virulence, it is important that spores
73 grown in the lab are representative of the spores in the clinical environment. If the spore preparation
74 method alters the outer spore layer, the spore properties could change significantly. For this reason,

75 we also sought to characterise the spore surface morphology depending on the method used to
76 produce the spores to see if it had an effect on their appearance and surface properties.

77

78 **2. Materials and Methods**

79 **2.1 Strains and growth conditions.** The clinical isolates of *C. difficile* used in this study are shown
80 in Table 1 and were obtained from the National Anaerobic Reference Unit, Cardiff, Wales. The
81 CD630 strain and its mutants by insertional inactivation were obtained from the laboratory of Prof.
82 Simon Cutting, University of Royal Holloway. The R20291, DS1748 and DS1813 originated from
83 the National Anaerobic Reference Unit, Cardiff, Wales. Unless otherwise stated, all organisms were
84 stored as spores at 4°C. Cultures were incubated at 37°C in a BugBox Plus anaerobic workstation
85 (Ruskin Technology Ltd., Bridgend, United Kingdom) using an 85% nitrogen, 10% carbon dioxide,
86 and 5% hydrogen gas mix.

87 **2.2 Media composition**

88 Wilson's broth [26] contained the following per litre: 90g Trypticase peptone, 5g Proteose peptone,
89 1 g Ammonium sulphate, 1.5 g Tris. The pH was adjusted to 7.4 following autoclaving. BHIS agar
90 [24] contained the following: BHI agar + 5g/l yeast extract + 0.1% L-cysteine. For CD630 structural
91 mutants, this agar was further supplemented with 5mg/ml erythromycin. Unless otherwise stated,
92 all reagents were purchased from Sigma Aldrich, Dorset, UK. Unless otherwise stated, the centrifuge
93 used in the purification steps was an Eppendorf 5417R centrifuge.

94 **2.3 Spore production and purification methods**

95 **Lawley's method.** The following method, based on the work of Lawley and colleagues [14], was
96 employed to produce *C. difficile* spores. To produce spores, a single colony harvested from a BHI
97 agar plate was used to inoculate 25ml of Wilson's broth, which was then incubated for 10 days at
98 37°C in anaerobic conditions.

99 To purify the spores, the cultured broth was centrifuge at 16,800 g for 15 min using a Beckman
100 Coulter J-20 centrifuge, the supernatant was discarded, and the pellet was resuspended in 1.5ml
101 distilled water. This washing step was repeated 4 more times using an Eppendorf 5417R centrifuge
102 and the final pellet was resuspended in 1.5ml PBS. The spore suspension was then subjected to
103 sonication for 90s using a tapered probe set at an amplitude of 35%, in a Soniprep 150 sonicator.
104 Following sonication, the sample was mixed with 1.5ml of 10% Sarkosyl and incubated for 1 hour
105 at 37°C with agitation. Samples were then pelleted at 3,400 g for 10 min and the pellets were
106 resuspended in 1.5ml of PBS + 0.125 M Tris buffer (with pH 8) + 10mg/ml lysozyme and incubated
107 overnight at 37°C with agitation. The suspensions were then sonicated again as described above, but
108 with 1% Sarkosyl instead of 10% Sarkosyl prior to the 1-hour incubation.

109 The suspensions were then layered onto a 50% sucrose solution and centrifuged at 3,400 g for 20
110 min. The pellets were resuspended in 2ml of PBS containing 200 mM EDTA, 300ng/ml proteinase
111 K + 1% Sarkosyl and incubated for 1 hour at 37°C with agitation. The suspensions were then layered
112 on 50% sucrose and centrifuged as described above. The resulting pellets were washed with sterile
113 distilled water (SDW) twice and finally resuspended in SDW and stored at 4°C.

114 **Sorg's method.** Described by Sorg & Soehnshein [6, 24], this method differs from that of Lawley
115 in that the spores are produced on agar rather than in broth and the purification process is less

116 complex. Bacteria were incubated in BHIS agar anaerobically at 37°C for 4 days. Following
117 incubation, cells were collected from the surface of the plate, using a 10µl inoculating loop and
118 suspended in 1ml SDW in a sterile Eppendorf tube. The suspension was incubated at 4° C overnight
119 and then centrifuged at 5000g for 5 min, the supernatant was discarded and the pellet was
120 resuspended in 1ml ice cold water. This washing step was repeated 4 times. The suspensions were
121 then layered onto a 50% sucrose solution and centrifuged at 3,400 g for 20 min and the pellet was
122 resuspended in 1ml ice cold water. The suspension was then centrifuged at 5,000 g for 5 min, and
123 the pellet was resuspended in 1ml ice cold water. This washing step was repeated 4 times. The
124 resulting final pellet was resuspended in SDW and stored at 4°C.

125 **Heeg's method.** The following method is based on the work of Heeg and colleagues [23]. Bacteria
126 were incubated on BHIS agar further supplemented with 250µg/ml cycloserine and 8µg/ml cefoxitin
127 anaerobically at 37°C for 4 days. Following incubation, cells were collected from the surface of the
128 plate, using a 10µl inoculating loop and suspended in 1 ml SDW in a sterile Eppendorf tube. The
129 suspension was incubated at 4°C overnight and then centrifuged at 16,000 g for 4 min, with the
130 supernatant and top layer of the pellet carefully removed after centrifugation. The rest of the pellet
131 was resuspended in SDW and the washing step repeated 10 times. The resulting final pellet was
132 resuspended in SDW and stored at 4°C.

133 **Counting viable spores.** To count the number of viable spores produced in all three of the above
134 methods, a serial dilution and drop count was used. The spore suspension was serially diluted,
135 mixing 10µl of the suspension with 10µl of SDW, with steps from 10⁻¹ to 10⁻⁸ dilution. From each
136 of the dilution steps, three 10µl drops were placed on a BHI agar plate supplemented with 0.1%
137 sodium taurocholate (Joshi et al., 2012). Based on the number of colonies which were seen after a
138 48-h incubation, the original concentration of viable spores was calculated.

139 **2.4 Visualisation of spores by light microscopy**

140 A standard Gram staining method was used to stain the spores [27]. 10µl of spore suspension was
141 placed on a glass slide and dried under a flame. The slide was subsequently flooded in crystal violet,
142 Gram's iodine and safranin for up to 30 seconds in each step. Slides were washed with water
143 following crystal violet and Gram's iodine flooding, and with ethanol following Safranin.
144 Vegetative cells, if any, were stained and would appear as long purple rods. A Leica DM2500
145 microscope was used to visualise the spores, using x1000 magnification and oil immersion. Spores
146 were then visualised using phase contrast settings in the microscope, with the slide under oil
147 immersion.

148 **2.5 Determining Spore Suspension Purity**

149 Spore suspensions were gram-stained as described above. The ratio of vegetative cells (determined
150 by gram staining) to spores (determined by phase contrast) was determined for 3 separate fields of
151 view for each sample. The fields of view were chosen to contain at least 100 objects (spores or cells),
152 but without clumping which could disrupt counts.

153 **2.6 Visualisation of spores by Scanning Electron Microscopy (SEM)**

154 A 10 µl aliquot of the purified spore suspension from stock was dried on a glass slide. The slide was
155 then coated with a film of Gold-Palladium using the Agar Scientific Sputter Coater in three 15-
156 second coating runs, using argon plasma. SEM images were captured using a Zeiss 1540 Crossbeam
157 Scanning Electron Microscope using Inlens and SE2 imaging modes. Spore dimensions were

158 measured from SEM images using ImageJ software. To determine spore characteristics, 30
159 individual spores were used from at least 6 fields of view. The analysis of spore samples by electron
160 microscopy was undertaken in the Cardiff School of Engineering.

161 **2.7 Hydrophobicity assay**

162 The Microbial Adhesion to Hydrocarbons (MATH) test was employed to determine the
163 hydrophobicity of spores examined in this study [28]. A 3ml spore suspension in distilled water with
164 an OD₆₀₀ 0.4-0.6 was prepared in a McCartney bottle. OD measurements were made using an
165 Ultrospec 1100 *pro* UV/Visible spectrophotometer, (Biochrom, Cambridgeshire, UK). A 300µl
166 aliquot of hexadecane was then added to the suspension and vortex mixed (VortexGenie, Fisher
167 Scientific, UK) for 1 min at room temperature. The mixture was incubated for 15 min at room
168 temperature, allowing the layers to separate, with hexadane and hydrophobic components of the
169 suspension rising to form to form the top layer, while the aqueous layer settled below with the
170 hydrophilic components of the suspension. After this, the OD of the aqueous (bottom) layer was
171 measured. The resulting decrease in the OD of the aqueous layer, compared to the the OD before
172 the addition of hexadecane, was recorded.

173 **2.8 Sonication**

174 To determine the feasibility of removing the exosporium, we adapted the methods of Escobar-Cortes
175 [29] and by Alyousef [30] which showed that intense sonication can remove the exosporium. For
176 this investigation, 1ml spore suspensions with 10⁷ spores each, were treated with 16 sonication
177 cycles of sonication of 15µm amplitude for 50 sec each, and cooling on ice for 1 min between each
178 cycle (Soniprep 150 sonicator, exponential probe).

179 **2.9 Statistical tools**

180 For simple statistical tools, including t-test and standard deviation, Microsoft Excel was used. For
181 statistical tests where more than two data sets were compared, ANOVA tests were done using
182 Graphpad Prism 5.0.

183

184 **3. Results**

185 **3.1 Selection of the optimal growth and spore purification method**

186 To support our studies to characterise differences in spore structure between different clinical
187 isolates of *C. difficile* we first sought to identify a method which maximised spore yield and purity.
188 As can be seen from Figure 1, both the Lawley and Sorg methods yielded similar levels of purified
189 spores at 10⁷–10⁹ cfu/ml (per 25 ml of broth for Lawley’s method and per agar plate with 25 ml of
190 agar for Sorg’s method) while the Heeg method only yielded 10³–10⁴ cfu/ml per 25 ml agar plate.

191 To determine the reason for the low spore cfu when using the Heeg method, we compared spore
192 numbers at different stages of the culture and purification process. Both the Lawley and Sorg
193 methods produced spore counts ranging from 10⁷–10⁹ cfu/ml following primary culture and lost
194 47.5% and 40% of these spores, respectively, following purification. The final spore suspensions
195 were of high purity, with more than 99% spores (Figure 2 A-B). In contrast, the Heeg method yielded
196 only 10⁴ cfu/ml upon primary culture of which 52.3% were lost during purification. The final
197 suspension, when observed by light microscopy, was mostly composed of vegetative cells and debris

198 (Figure 2C). Overall, there was no statistically significant difference in percentage spores lost
199 between the 3 methods ($p=0.09$). The results suggest that the poor spore yield using the Heeg method
200 was due to a failure of the individual bacterial strains to produce spores in the cycloserine- and
201 cefoxitin-enriched agar.

202 **3.2 Physical dimensions of spores**

203 The physical dimensions of the spores produced using the different production and purification
204 methods varied markedly, ranging from 1.2 μm to 2.6 μm in length and from 0.7 μm to 1.15 μm in
205 width (Figure 3). To determine if any of these results were significantly different, we compared the
206 results obtained for each isolate produced using the Lawley and Sorg methods using Tukey's
207 multiple comparison test. Overall, spores produced using the Lawley method were larger than the
208 spores produced with the Sorg method. This difference was not significant for the strains DS1813
209 ($p > 0.05$) and CD630 but was significant for strains 1748 ($p = 0.011$ for length) and R20291
210 ($p < 0.001$ for length).

211 To determine if the different spore production and purification methods had an effect on surface
212 properties, spores produced by different clinical isolates of *C. difficile* were visualised by SEM. As
213 can be seen from Figure 4, spores of different clinical isolates produced using the same method
214 varied in their appearance. It is notable that despite belonging to the same "hypervirulent" 027
215 ribotype, the spore forms of DS1813 and R20291 differed markedly in appearance (Figure 4 C-F).

216 **3.3 Effect of the spore purification method on spore structure**

217 Next, we determined if the different spore production and purification methods had an effect on the
218 overall appearance of spores of the same clinical isolate. While the different methods had no visible
219 effect on the structure of spores produced by DS1813 this was not the case for R20291 spores.
220 Spores of the R20291 strain purified using the Sorg method differed in structure from those purified
221 using the Lawley method. The majority of spores (97%) purified using the Sorg method were
222 surrounded by a loose layer. In contrast only 44% of the spores produced using the Lawley method
223 were surrounded by a loose layer, likely due to the extra sonication and proteinase steps in the
224 purification method. We also observed what appeared to be filaments, approx. 80 nm in width,
225 extending from the spore surface (Figure 4F). The morphology of spores produced by DS1813 also
226 varied depending on the production and purification method. 70% of the spores purified with the
227 Sorg method had "ridge" structures as seen on Figure 4C. In spores purified with the Lawley method,
228 only 21% showed these features (data not shown). For DS1748, the ratio of pineapple-like spores
229 was higher when produced using the Sorg method (75% of those observed), and higher for DS1748
230 when produced using the Lawley method (99%). The ratio of spores with a bag-like layer was higher
231 for both R20291 (98%) and CD630 (86%) when using the Sorg method.

232 To determine if spore coat protein mutants of CD630 differed in their appearance when produced
233 using the two methods they were also subjected to SEM. However, we observed no obvious
234 differences in the appearance (Figure 5) of the spore form of the various BclA and CotE mutants
235 when compared to the parent strain.

236 **3.4 Sonication of *C. difficile* strains**

237 To obtain data on how different strains are changed by sonication and whether the sonication step
238 in Lawley's method might have affected spore surface, the four strains purified with the Sorg method

239 were sonicated and then observed using SEM. Sonication resulted in the loss, to varying degrees, of
240 the outermost surface features of the spores of all four clinical isolates (Figure 6). Sonication of the
241 630 and R20291 strains resulted in the removal of the loose layer which surrounds the untreated
242 spore (Figure 4, blue arrows) in 49 and 85% of the total observed spores respectively. In the case
243 of DS1813 the disruption was less marked with the loss of surface “ridge” structures from 39% of
244 the spores while spores of DS1748 saw the least disruption with only 20.3% change (Figure 7).
245 Sonication of DS1813, CD630 and R20291 yielded spores similar in appearance to those produced
246 using the Lawley method. This was not the case for the 1748 strain, where the changed were stripped
247 of the outermost layer, which could be observed separately from the spore (see example Figure 8).

248 **3.5 Hydrophobicity of *C. difficile* spores**

249 To determine the effect of the individual purification methods on spore properties, the commonly
250 used Microbial Adhesion to Hydrocarbons (MATH) test was used. The relative hydrophobicities
251 (RH) of the different strains are shown on Figure 7. Comparison of the RH values of the spores
252 produced using the Lawley method revealed no statistically significant difference (ANOVA $p=0.67$),
253 suggesting that all four strains possessed similar levels of hydrophobicity.

254 In contrast, when the spores from the same clinical isolates were produced and purified using the
255 Sorg method, we observed a statistically significant difference in RH values across all of the strains
256 (ANOVA $p=0.0001$), with the spores of the DS1748 and DS1813 strains having the highest
257 hydrophobicity and the R20291 strain having the lowest.

258 Sonication of the Sorg method-produced caused a significant reduction in RH values of all isolates
259 when compared to the unsonicated Sorg spores ($p<0.0001$), suggesting that sonication had caused
260 changes in spore surface properties. For the DS1813, CD630 and R20291, the sonicated strain also
261 had lower RH values than the spores purified with Lawley method.

262

263 **3.6 Structural mutant hydrophobicity**

264 The hydrophobicity of the spores of the CotE and BclA insertional inactivation mutants was
265 determined and compared to that of clinical isolates. Previous studies [32] have shown that BclA
266 mutants have altered spore hydrophobicity, but the difference in methods made comparisons with
267 isolates in our study not possible, so the hydrophobicity measurements were done with spores
268 purified using the Sorg method.

269 As can be seen from in Figure 9, the knockout strains do show different hydrophobicities compared
270 to the isogenic strain of CD630. The differences between CD630 strain mutants are significant
271 overall ($p=0.0004$). The greatest differences were seen in the CotEn and BclA3 strains. CotEn is
272 significantly higher in hydrophobicity than other strains ($p=0.001$). BclA3 was the lowest in
273 hydrophobicity than other strains ($p=0.006$).

274

275 4. Discussion

276 Ideally a spore production method should minimize the damage to the final spores so that they mirror,
277 as much as possible, the native form encountered in the context of disease. This is particularly
278 important for a bacterium such as *C. difficile* which as a species exhibits markedly genomic plasticity
279 due to horizontal gene transfer [33]. Thus, while isolates may share the same core genes and thus
280 belong to the same genotype (depending on how it is defined) they may differ markedly in
281 characteristics such as spore surface structure.

282 When we compared the spores produced by *C. difficile* belonging to the same hypervirulent ribotype
283 (027) we observed marked difference in spore ultrastructure which was affected by the spore
284 production method. R20291 spores produced two distinct morphotypes, smooth and surrounded by
285 a bag-like layer. The relative proportion of each morphotype was influenced by the spore production
286 method. While the majority of R20291 spores produced using the Sorg method were surrounded by
287 a bag-like layer this number reduce to less than half using the Lawley method suggesting some form
288 of physical disruption.

289 A similar change in the morphotype frequency was observed with DS1813 (Ribotype 027) and
290 CD630 (Ribotype 012) suggesting that some aspect of the Lawley purification method may have an
291 adverse effect on structural integrity. Unlike the Sorg method the Lawley purification protocol
292 includes a sonication step. Independent sonication of the spores used in this study resulted in a higher
293 proportion of smooth spores suggesting that this process may have contributed the shift in spore
294 morphotype observed using the Lawley method. It must be noted that despite the sonication and
295 loss out other layers feature, spores isolated using the Lawley method are slightly larger. The larger
296 size of the spores produced during the Lawley method could be due to both differences in the media
297 composition and due to the fact that it was a liquid culture, as opposed to growth on an agar.

298 Pineapple-like structures on the surface of *C. difficile* spores were reported previously [39], where
299 it was thought to be a common feature among *C. difficile* spores in general. These features may also
300 be related to the “bumps” observed using TEM on the surface of the spores of TL176, TL178 and
301 R20291 strains of *C. difficile* which have been linked to the “thick” exosporium morphotype [35].
302 However, unlike Pizarro-Guajardo et al., we did not observe the presence of short hair-like structures
303 surrounding our spores but did observe long hair-like features projecting from R20291 spores
304 produced using the Lawley method. Overall, this illustrates how varied *C. difficile* spore morphology
305 is varied between different clinical isolates and can present different phenotypes that can depend on
306 the isolate and the methods used to culture and purify them. The individual results cannot be
307 compares due to the major difference in methods of growth and isolation of *C. difficile* (summarised
308 in Table 2).

309 As was the case for spore morphology, spore hydrophobicity also varied depending on the clinical
310 isolates and the methods used to produce them. While spores from different isolates produced using
311 the Sorg method varied markedly no such differences was seen when the spores were produced
312 using the Lawley method. Sonicated of Sorg produced spores resulted in a decrease in the
313 hydrophobicity of all of the isolates supporting the supposition that this step in the Lawley
314 purification process could be responsible for these differences. This is line with previously reported
315 results for the CD630 strains of *C. difficile* [29]. In this study we confirm this is also the case for

316 other strains of *C. difficile* and for “pineapple-like” spores the outermost layer can be observed
317 separately from the rest of the spore. This opens up possibilities for further study of the composition
318 and structure of this layer alone.

319 Why is this important? Studies have shown that *C. difficile* R20291 spores with a defective outer
320 bag like adhere more efficiently to Caco-2 cells than their intact counterparts suggesting a potential
321 role in adherence to epithelial surfaces and in the transmission of CDI [34]. Removal of the outer
322 layer has also been shown to increase the ability of *C. difficile* spores to germinate [35]. If we are to
323 fully understand the contribution of individual spore structures to the virulence of a particular isolate
324 of *C. difficile* it is important, given the inherent diversity of the species, to employ methods which
325 do not alter the structural integrity of the spores we are attempting to study.

326 For this reason, in this study we compared the appearance and hydrophobicity of spores produced
327 using the Sorg method. The bag-like layer surrounding spores of R20291 and to a lesser extent
328 CD630 were similar in appearance to that seen surrounding spores of the *Bacillus cereus* family
329 [25]. In *B. cereus* the layer is hexagonal in structure around 8 nm in diameter and is composed of
330 two proteins, *CotY* and *CotE* [21], with filament of a glycoprotein called *BclA* protecting from the
331 surface [36].

332 The composition of the *C. difficile* outermost layer is not as well characterised as that of *B. cereus*,
333 but in the case of R20291 this layer is based on two cysteine-rich proteins; *CdeC* and *CdeM* [37].
334 They are similar in nature to the *B. subtilis* proteins *CotY* and *CotZ*. As with *B. cereus*, the filaments
335 projecting from the surface of *C. difficile* spores are composed of *BclA* homologs. In both *B. cereus*
336 and *C. difficile* the filaments have been implicated in the attachment of the spore to bind to intestinal
337 cells [38]. This attachment may be aided by the flexible nature of the bag-like layer which assists
338 by maximising the contact area between the spore and cell surface.

339 The second major outer spore structure observed in this study, the “pineapple-like” layer, had
340 previously been reported to be a common feature of *C. difficile* spore isolates [39]. In TEM studies
341 this layer was observed to be electron dense and tightly bound to the inner spore. The structure of
342 this layer to date has not been characterised.

343 It is possible that the “pineapple-like” structure may contribute to hydrophobicity, as they increase
344 surface roughness and decrease wetting. Solid features of similar size to the “bumps” on the spore
345 surface (125 nm diameter) are present on natural hydrophobic surfaces were they were shown to
346 contribute to the hydrophobic properties [40].

347 In an attempt to determine the contribution of individual known spore structural proteins to spore
348 surface morphology, we compared the appearance of wildtype CD630 spores to variants lacking
349 individual structural spore proteins. Our failure to observe the structural deficiencies described in
350 earlier work is probably a reflection of the fact that we employed SEM rather than TEM to visualise
351 the spores [30, 31]. While SEM does not provide the level of magnification and resolution which
352 can be achieved using TEM it does provide more information about the 3D shape of the structure
353 which is extremely useful when attempting to characterise the surface architecture of spores.

354 In contrast to the data obtained from electron microscopy, our hydrophobicity results support the
355 hypothesis [33] that the loss of the *C. difficile* homologs of BclA have an effect on surface chemistry
356 and are similar to previously reported results. It is possible that the removal of this protein unmasked
357 polar groups on the spore surface, without damaging the spore integrity. In contrast, the loss of the
358 CotE homolog also failed to impact on hydrophobicity suggesting that the wild type protein is not
359 surface exposed.

360 In conclusion, we have seen a variety we observed significant variation in the spore morphology of
361 clinical isolates of *C. difficile*, due in part to the methods used to sporulate and purify them. Three
362 distinct spore morphotypes were identified and the differences in these morphotypes were connected
363 to different spore hydrophobicity. Sonication in particular can significantly change spore appearance
364 and properties by removing the outermost layer of the spore. Finally, this work highlights the need
365 for a common “standard” growth and purification method for *C. difficile* spores to allow for
366 comparisons of results obtained by different research teams.

367 **5. Acknowledgments**

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371 operation.

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373 providing the *C. difficile* CD 630 knockout mutant strains.

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498 **8. Tables**

499

500 Table 1. Strains of *C. difficile* used in this study.

Strain	Ribotype	Description
R20291	027	Hypervirulent ribotype, Stoke-Mandeville strain [17]
DS1813	027	Relatively high hydrophobicity of 87%, no visible exosporium [17]
DS1748	002	Relatively low hydrophobicity of 12% [17]
CD630	012	The first isolate of <i>C. difficile</i> to be genome sequenced [14]
CD630 BclA1 ⁻	012	Deletion mutant of BclA homolog 1 [31]
CD630 BclA2 ⁻	012	Deletion mutant of BclA homolog 2 [31]
CD630 BclA3 ⁻	012	Deletion mutant of BclA homolog 3 [31]
CD630 CotE ^{N-}	012	N-terminal deletion mutant of spore coat protein CotE [32]
CD630 CotE ^{C-}	012	C-terminal deletion mutant of spore coat protein CotE. [32]

501

502 Table 2. Methods used in studies observing “pineapple-like” *C. difficile* Spores

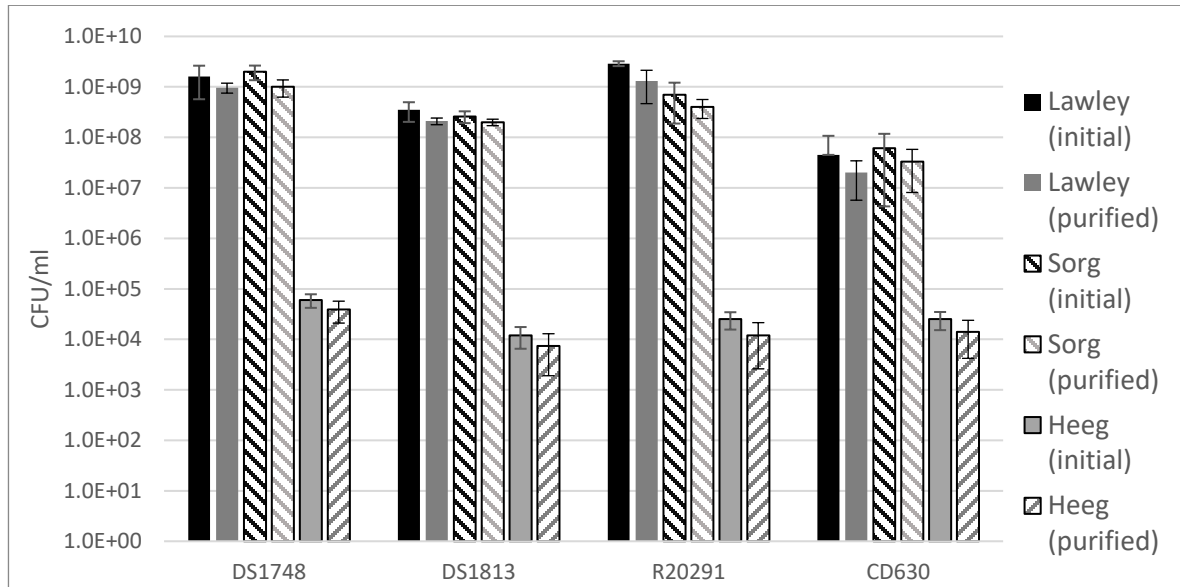
Study	Imaging	Source of strains	Incubation media	Incubation Duration	Purification of Spores
Rabi et al., 2017	SEM and TEM	O'Connor et al., 2006 [41] Carter et al., 2007 [42]	Trypticase Yeast broth + with 0.1% sodium thioglycolate	10 days	Manual removal of top layer of pellet over multiple washes
Girinathan et al., 2017 [43]	TEM	Stabler et al., 2009 [44]	BHIS Agar	4 days	Density Gradient with 50% sucrose
Pizarro-Guajardo et al., 2016	TEM	Laboratory strains	3% Trypticase soy -0.5% yeast extract agar	5 days	Density Gradient with Nicodenz
This study	SEM	National Anaerobic Reference Unit, Cardiff, Wales	BHIS Agar	4 days	Density Gradient with 50% sucrose
	SEM		Wilson's Broth	10 days	Density Gradient + Sonication + Proteinase K

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504

505 **9. Figures and Legends**

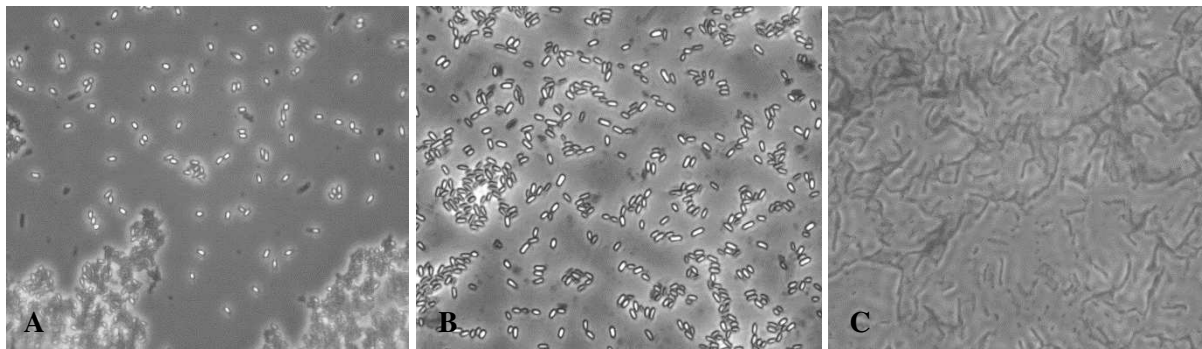
506



507

508 Figure 1. The initial yield of spores after growth in media and final yield of purified spores of clinical
509 isolates of *C. difficile* obtained using three different production and purification methods (n=3).

510

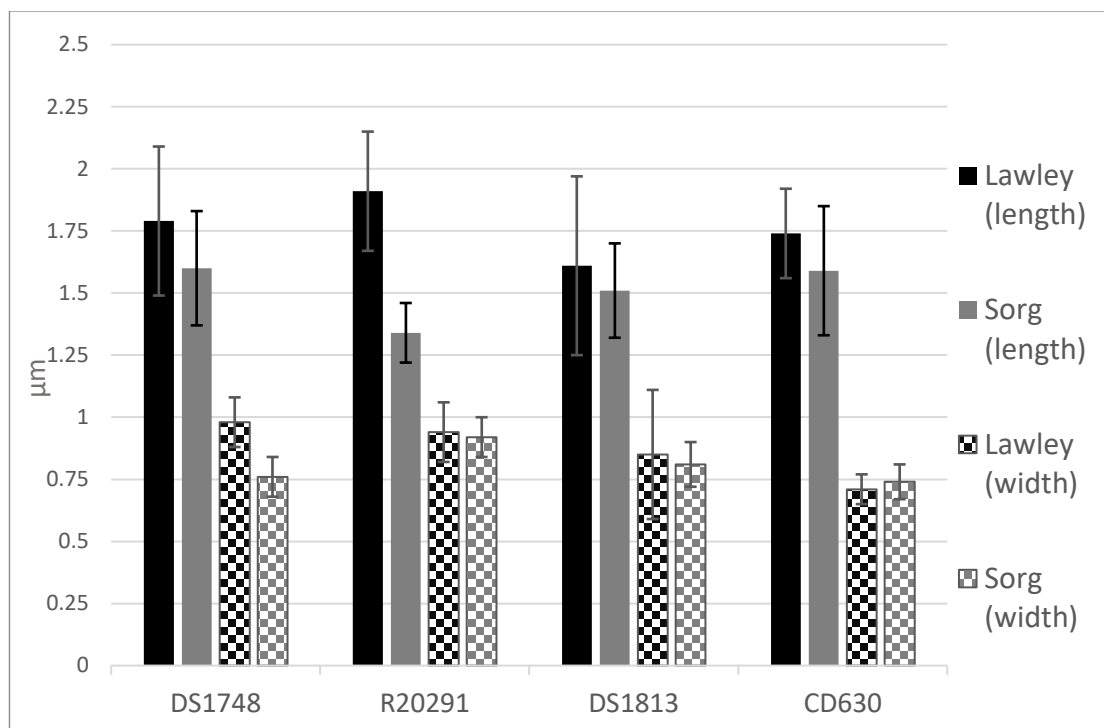


511

512 Figure 2. Phase contrast microscopy of *C. difficile* DS1748 spore suspension following purification
513 using the Lawley method (A), Sorg method (B) and Heeg method (C). When purified with Lawley
514 method or the Sorg method, the suspension is composed of mostly phase bright spores which can
515 be clearly observed. However, with the Heeg method the suspension is mostly composed of
516 vegetative cells and debris, while spores are not visible under phase contrast.

517

518



519

520 Figure 3. The effect of production and purification methods on the physical dimensions of spores of
 521 four *C. difficile* isolates determined from SEM micrographs. 30 spores of each strain were measured.

522

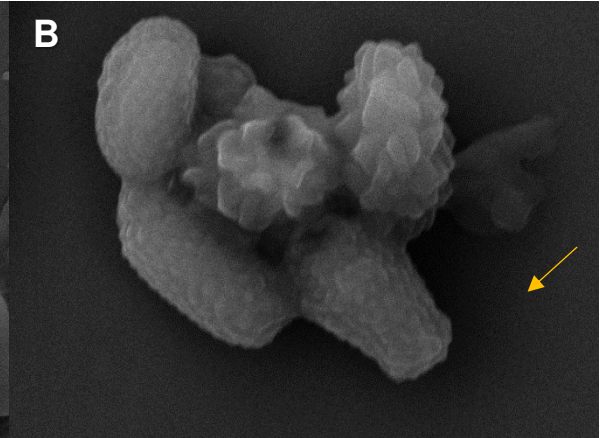
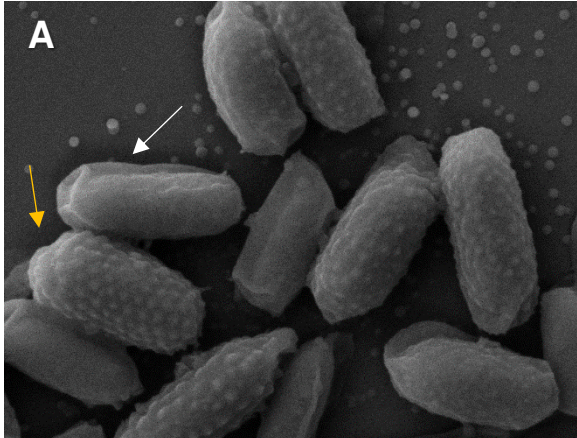
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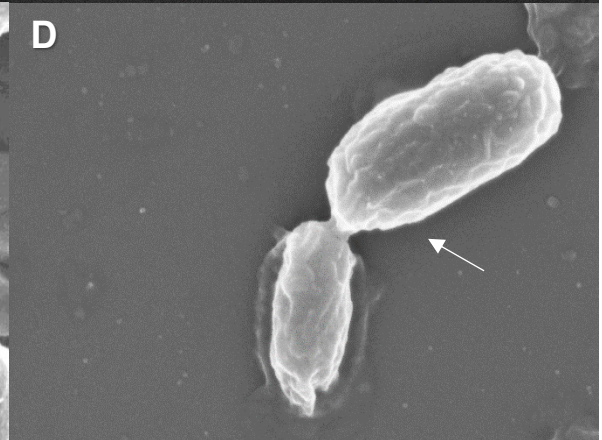
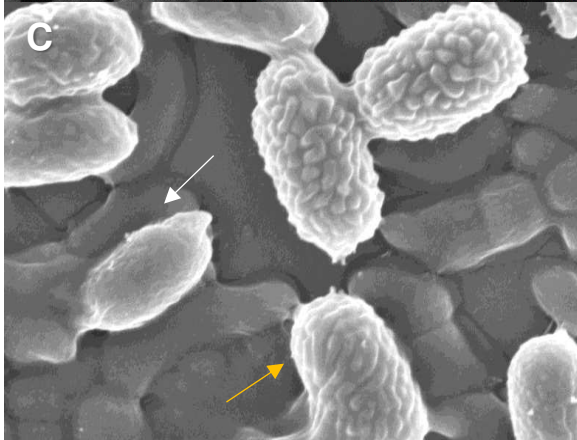
Sorg Method

Lawley Method

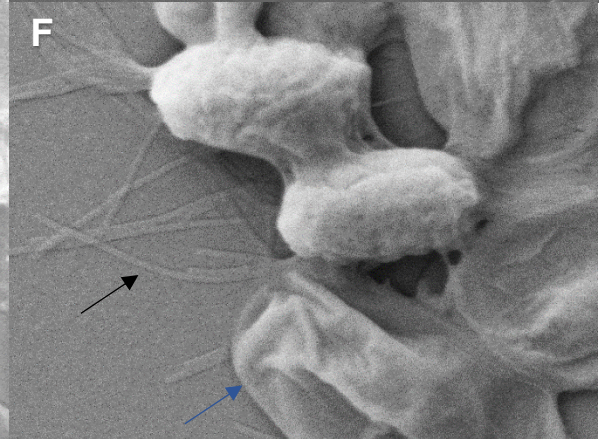
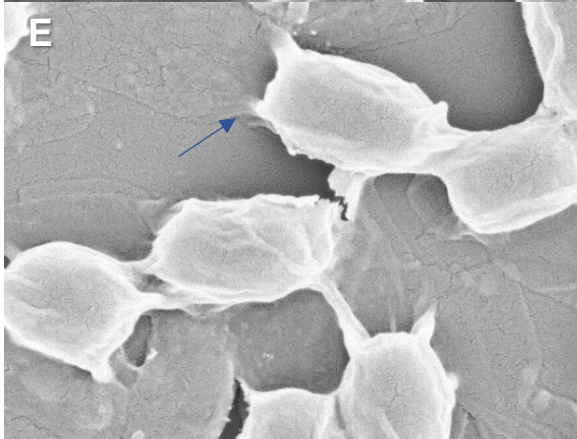
DS1748



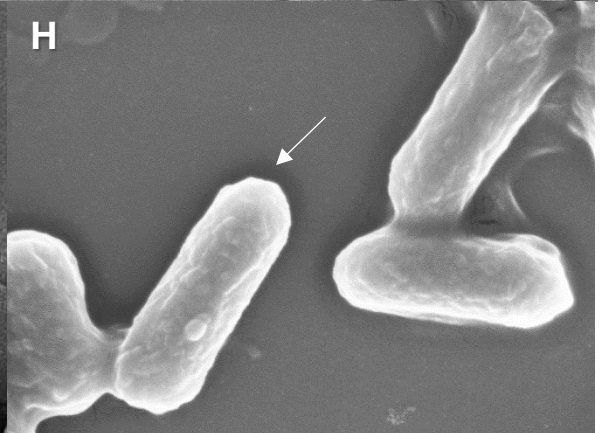
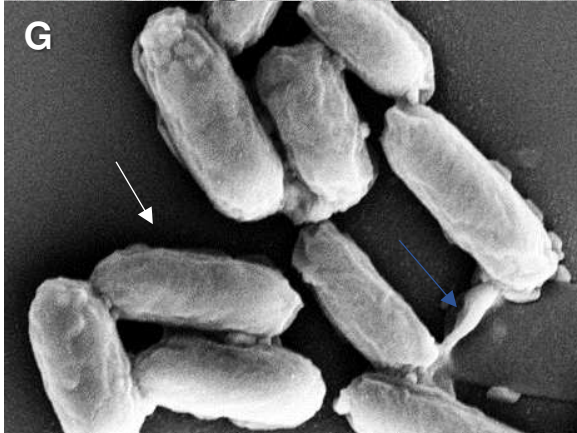
DS1813



R20291

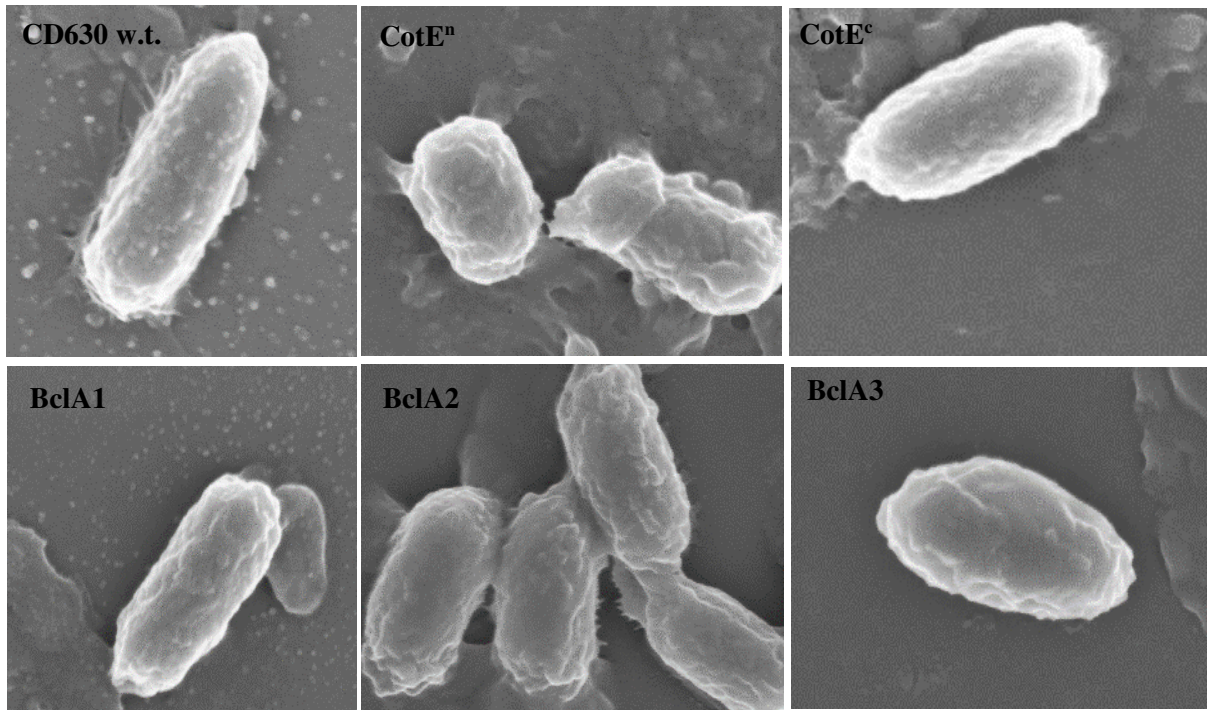


CD630



526 Figure 4. Surface morphotype variability of *C. difficile* depending on clinical isolate (rows) and
527 method of spore growth and purification (column). Arrows show examples of different morphotypes.
528 Orange arrows show examples of “pineapple-like” spores. Blue arrows show spores with an
529 associated with a bag-like layer. White arrows show plain smooth spores. Black arrows show
530 filaments extending from spores. Images are representative of a set of 30 fields of view for each
531 strain.

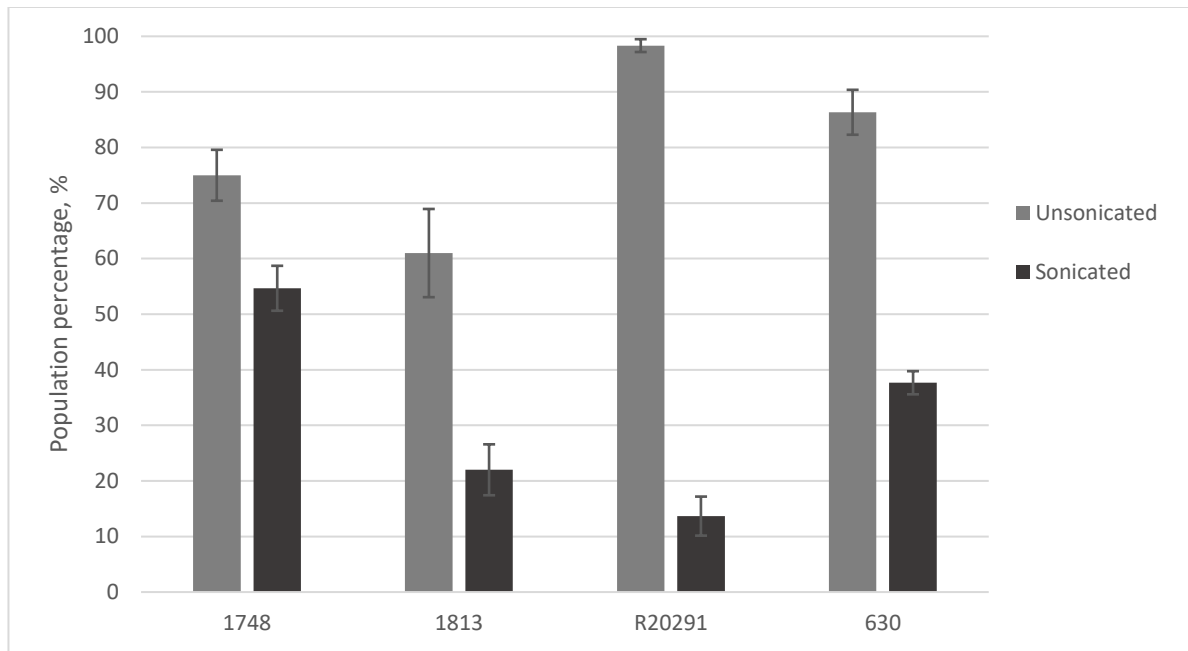
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533

534 Figure 5. Spores of mutant strains of *C. difficile* CD630. No clear differences in surface feature was
535 seen. Images are representative of a set of 10 fields of view for each strain.

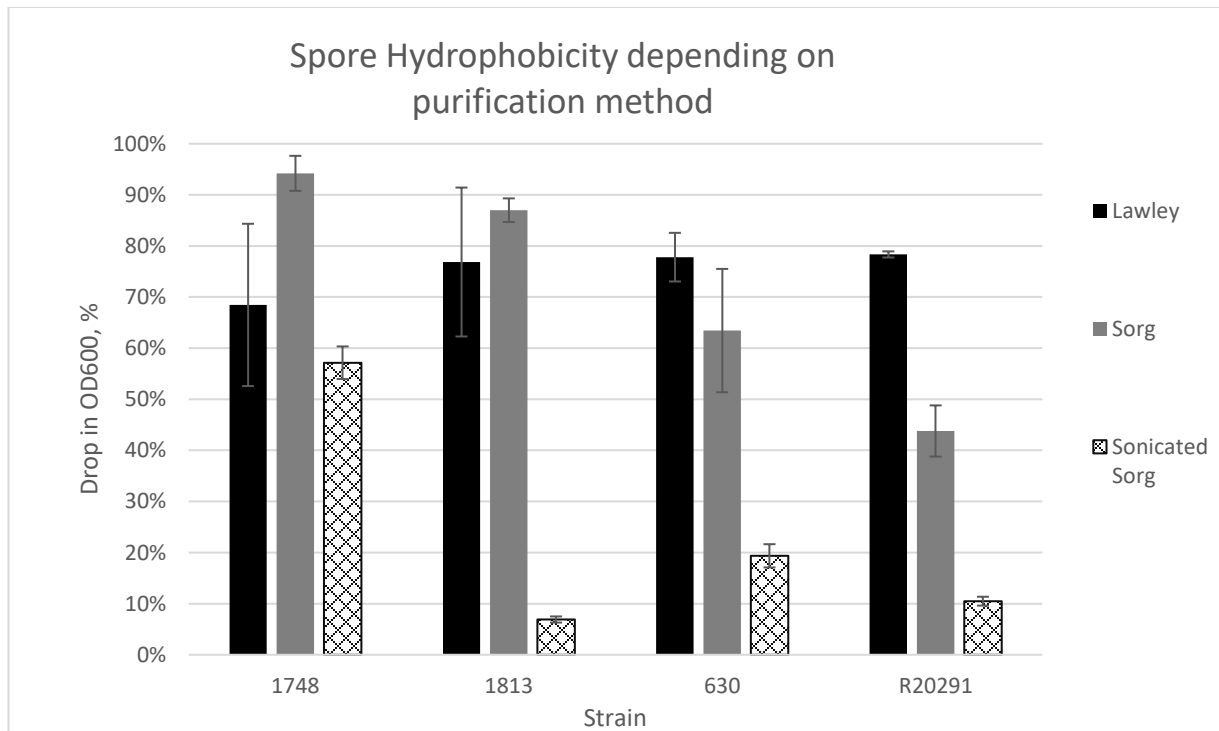
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538 Figure 6. Change in the relative proportion of spores with a recognisable feature (pineapple shape
 539 or loose exosporium) with and without intense sonication (n=3, 50 spores per repeat). The reduction
 540 is significant for all 4 strains (p=0.005 for 1748; p=0.004 for 1813; p<0.001 for R20291; p<0.001
 541 for 630).

542

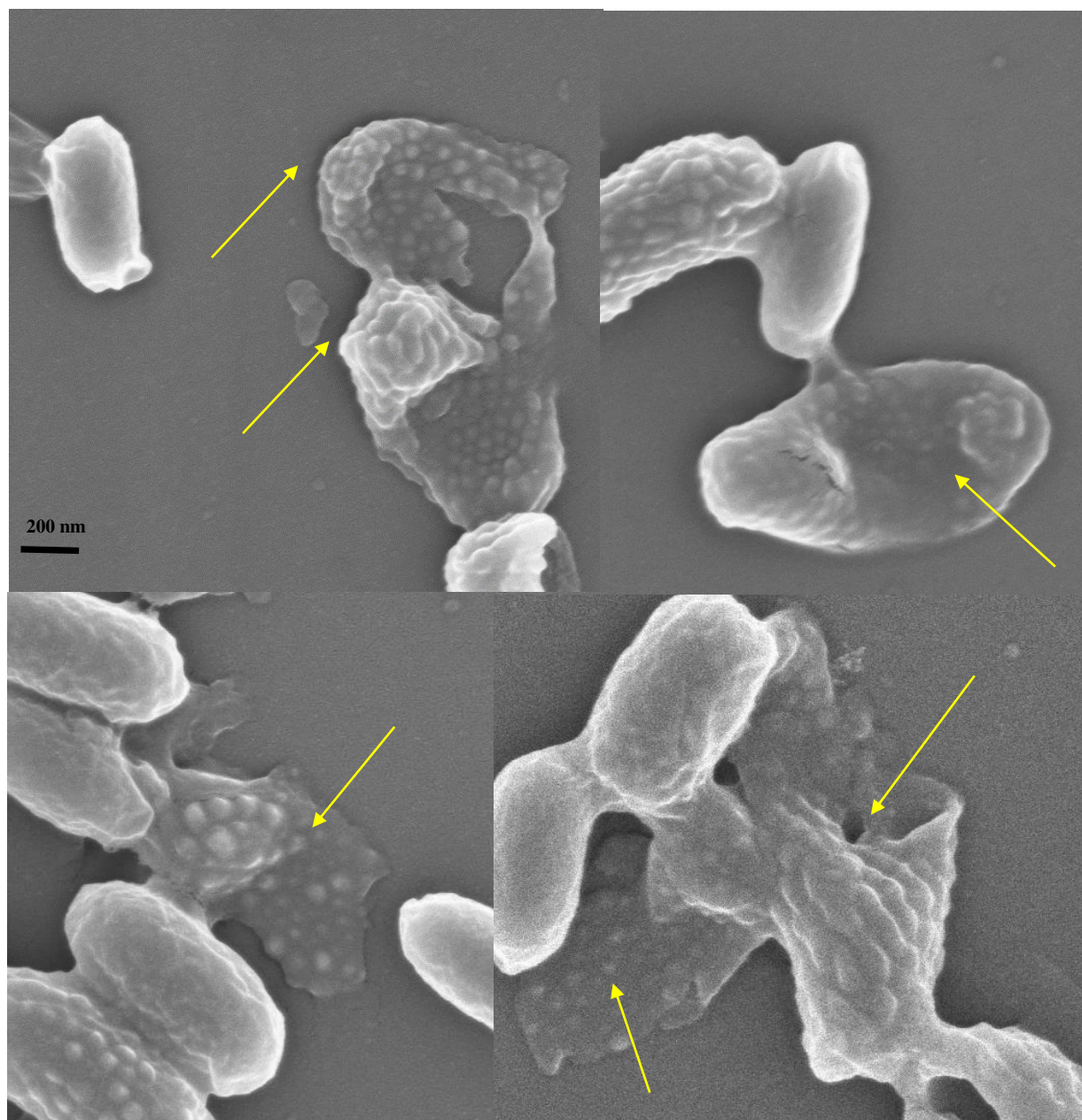


543

544 Figure 7. Relative hydrophobicities (RH) of spores of four clinical isolates *C. difficile* spores
 545 produced and purified using three different methods.

546

547

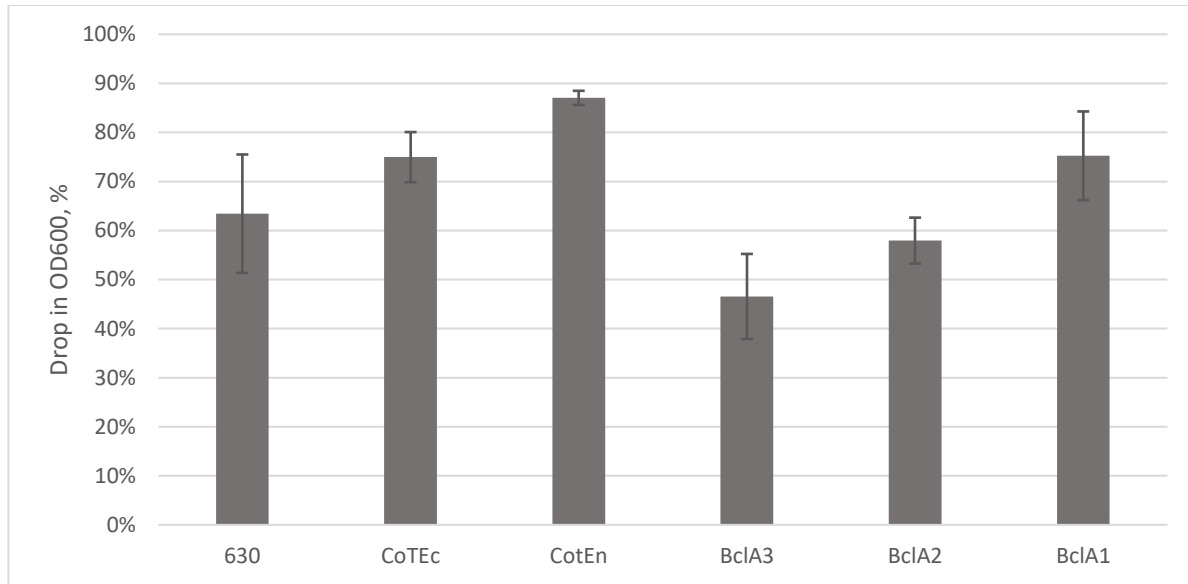


548

549 Figure. 8. Fragments of the outer “pineapple” layer of sonicated DS1748 spores are indicated by
550 yellow arrows, and a “smooth” 1748 spore can see also be seen. These images are representative of
551 10 fields of view.

552

553



554

555 Figure. 9. The relative hydrophobicity of spores of *C. difficile* strain CD630 and its mutants deficient
556 in a structural protein. All spores produced and purified using the Sorg method (n=3 replicates of
557 each strain).

558