Assessment of chemical methods in the removal of the spore coat and exosporium layers of *Clostridioides difficile* Spores.

Javier Sanchez[1], Alba Romero-Rodriguez[2#], Scarlett Troncoso-Cotal[2⊗], and Daniel Paredes-Sabja[2,3*]

[1]Department of Biology, Texas A&M University, College Station, Texas, U.S.A.

[2]ANID – Millennium Science Initiative Program – Millennium Nucleus in the Biology of the Intestinal Microbiota, Santiago, Chile.

[3]Department of Biology, Texas A&M University, College Station, Texas, USA, dparedes-sabja@bio.tamu.edu

Present address: Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas Universidad Nacional Autónoma de México, Ciudad de México.

⊗ Present Address: Department of Cellular and Molecular Biology, Pontificia Universidad Católica de Chile.

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*Corresponding Author. Dr. Daniel Paredes-Sabja, Department of Biology, Texas A&M University, College Station, TX, 77843, USA. E-mail: dparedes-sabja@bio.tamu.edu

Abstract

Clostridioides difficile spores are essential for initiation, recurrence and transmission of the disease. The spore surface layers are composed of an outermost exosporium layer that surrounds another proteinaceous layer, the spore coat. These spore surfaces layers are responsible for initial interactions with the host and spore resistance properties contributing to transmission and recurrence of CDI. During spore-development, assembly of both layers is tightly interconnected thus studying the surface is essential for understanding the assembly of these layers and identification of potential targets for therapeutics. Several spore coat /exosporium extraction methods have utilized different extraction procedures making comparison across studies difficult and their impact on spore surface layer properties remains unclear. Here, we tested how commonly used chemical methods remove the spore coat /exosporium layers, analyzing treated-spores by phase contrast microscopy, transmission electron microscopy, western blotting, and lysozyme-triggered germination to functionally characterize the extraction efficiency of these treatment on these layers. Our results provide a systematic analysis and offer a platform for future spore coat and exosporium-related studies.

Introduction

Clostridioides difficile (*C. difficile*) is a Gram-positive anaerobe that has become a leading cause of antibiotic associated diarrhea in developed countries[1, 2]. Treatment against *C. difficile* infections (CDI) resolve 95 % of primary cases; however, ~15 – 30 % of recovered CDI patients develop subsequent recurrent episodes of CDI, which result in mortality rates increasing up to 30%[3]. Although two major clostridial toxins, TcdA and TcdB, are necessary for disease manifestation[4], the production of *C. difficile* spores during infection is essential for recurrence[5, 6].

C. difficile spores are metabolically dormant and naturally resistant to antibiotics, heat, ethanol, and various cleaning agents[6, 7]. *C. difficile* spores are made by a series of concentric layers that assemble under the control of the mother cell developmental program, specifically under the RNA polymerase sporulation-specific sigma factors, SigE and SigK [6, 7]. The outermost exosporium layer of *C. difficile* spores play important roles in infection, recurrence and interaction with host molecules [8-12]. Importantly, during late stages of sporulation development, *C. difficile* forms two distinctive spores that differ in the thickness of the exosporium morphotype, while the underlying spore coat remains identical [6, 13, 14].

Both, the spore coat and exosporium of *C. difficile* spores are proteinaceous layers made of > 50 and ~ 10 - 20 proteins, respectively[15, 16]. Both layers reassemble a highly crosslinked protein matrix that can withstand various environmental insults[7]. Numerous studies on the spore coat and exosporium layer of *C. difficile* spores have varied in the methods to extraction of spore coat and exosporium layers [10, 15-23]. Because these chemical methods dissociate the entire proteinaceous spore surface layers, they are thought to extract the spore coat and exosporium layers, at least in

63 *C. difficile*[10, 15-23]. Early work utilized Lithium dodecyl sulfate sample buffer – 10% 2-
64 mercaptoethanol followed by heating at 90ºC for 10 min to remove the spore coat and exosporium
65 layers of *C. diffiicle* 630 spores [23]. Three additional methods have been utilized to remove these
66 extracts. For example, follow-up work utilized 2 X Laemmli buffer (4% SDS, 10% 2-
67 mercaptoethanol) followed by incubation for 10 min at 100 ºC [10, 15, 17]. Several proteomic studies
68 used a lysis buffer with 6 M urea, 5 mM DTT in ammonium bicarbonate at pH 8.0 followed by
69 bead beating at room temperature [16, 21, 22]. Finally, a blend of 8 M urea, 2 M thiourea, 5% (w/v)
70 SDS, 2% 2-mercaptoethanol, denominated EBB buffer, followed by incubation for 20 min at 95ºC
71 has also been used extensively mainly in sporulating cultures [18-20]. Although different in their
72 components, most of these approaches use: i) a chaotropic agent, urea or urea + thiourea to disrupt
73 hydrogen bonds and results in the dissolution of hydrophobic residues within the protein[24]; ii) SDS
74 to denature and solubilize proteins; iii) a reducing agent which can be 2-mercaptoethanol or
75 dithiothreitol. Despite the well use of these different chemical extraction methods, it is unclear
76 whether the spore coat and exosporium layer are efficiently removed through chemical methods,
77 commonly employed in *C. difficile* spore-research.
78
79 Efficient fractionation of the spore surface layers is detrimental to understand the localization of
80 spore surface constituents in each of these layers. In this regard, we demonstrated that enzymatic
81 digestion with proteinase K or trypsin, or a mechanical treatment with sonication, efficiently
82 removed only the exosporium layer of *C. difficile* spores [15]. This allowed the identification of the
83 exosporium proteome in spores of strain 630 [15]; however, a similar validation with chemical
84 extraction methods has not been conducted. Therefore, to improve our tool-box of methods to
85 study *C. difficile* spore architecture and composition, in this work, in this work, provide a

86 systematic analysis of how two chemical methods (i.e., USD and EBB); that are commonly
87 employed to remove the spore coat /exosporium extracts for immunoblotting analysis, remove
88 these layers and impact the functional aspects of *C. difficile* spores. In additional, Laemmli buffer
89 was tested for its efficiency to extract alone when compared to USD and EBB. By utilizing two
90 exosporium markers (i.e, CdeC and CdeM)[10, 25, 26], and the the spore cortex lytic enzyme SleC as
91 a marker for the peptidoglycan-spore coat interface [27, 28], we monitor the efficiency of extraction
92 of these layers through immunoblotting and transmission electron microscopy.
93

94 **Materials and Methods**

95 **Spore Preparation and Purification.**

96 *C. difficile* strain R20291 was grown in Brain Heart Infusion broth (BHIS) (Difco) supplemented
97 with 0.5% yeast extract and 0.1% cysteine anaerobically at 37˚C. Overnight cultures were grown
98 in BHIS broth with 0.1% sodium taurocholate and 0.2% d-fructose. Cultures were diluted to an
99 OD_{600} of 0.5 and 250 uL was plated on 70:30 sporulation media under anaerobic conditions for 5
100 days at 37˚C[29]. Sporulating cultures were collected from plates, resuspended in ice-cold distilled
101 water, and left in 4˚C overnight to allow for lysis of vegetative cells. After incubation, cells were
102 centrifuged and pellets washed with ice-cold distilled water 5 times followed by repeated
103 centrifugation and resuspension to remove vegetative cell debris. Spores were separated using a
104 60% sucrose gradient and centrifuged for 20 min at 14,000 rpm, spores were then washed with
105 ice-cold water to remove residual sucrose. Washing and sucrose gradient repeated until spores
106 reached 99% purity. Once pure, spores were quantified using a NeuBauer chamber and stored at -
107 80˚C until use at a $5x10^9$ spores/mL concentration.
108

109 **Spore Extraction Methods**

110 $1.5x10^7$ spores pelleted down and resuspended in 50 uL of buffer. Samples with EBB (EBB 8M
111 Urea, 2M Thiourea, 4% w/v SDS, 2% w/v β-mercaptoethanol) were boiled 10 minutes and spun
112 down at 15k rpm for 5 minutes [23]. Supernatant containing spore extract was saved and spore pellet
113 washed three times with distilled water to remove residual spore extract and buffer. Spores were
114 then retreated with EBB for a total of three extractions, with supernatant saved between each step.
115 USD (8M Urea, 1% w/v SDS, 50 mM DTT, 50 mM Tris-HCl pH 8) samples were treated for 90
116 minutes at 37˚C and pelleted down[30]. Supernatant was saved and spore pellet washed. 1˚ USD or
117 1˚ EBB treated spores were incubated with lysozyme for 37˚C overnight.

118

119 **Lysozyme Treatment**

120 *C. difficile* spores were treated with lysozyme to analyze the effects of the extraction buffers on
121 the integrity of the spore coat[29]. Spores ($1x10^7$ spores) were treated with 1mg/mL lysozyme in 25
122 mM phosphate buffer (pH 7.4) at 37˚C for 2 hours [10]. Samples were washed with distilled water
123 and analyzed under phase contrast microscopy. 200 spores were analyzed for germination and
124 binned for phase dark, phase grey, or phase bright. The data represents the results of three
125 independent experiments and spore sample preparations.

126

127 **Transmission Electron Microscopy and Analysis**

128 For transmission electron microscopy, we followed prior protocols with modifications[31].Briefly,
129 *C. difficile* spores were fixed overnight with 3% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2)
130 at 4˚C. Samples were centrifuged at 14k rpm for 5 minutes and supernatant discarded. They were
131 then stained with1% osmium tetroxide in 0.05M HEPES buffer (pH 7.4) overnight at 4˚C. Treated

132 samples were washed 5x with distilled water. The spores were dehydrated stepwise in 30%, 50%,
133 70%, 90% for 15 minutes respectively, followed by dehydration with 100% acetone 3 times for
134 30 minutes at each step. A small amount of acetone is left covering the sample to prevent
135 rehydration. Spore samples were embedded in modified Spurr's resin (Quetol ERL 4221 resin;
136 EMS; RT 14300) in a Pelco Biowave processor (Ted Pella, Inc.). Initially, 1:1 acetone-resin for 10
137 min at 200W-with no vacuum, 1:1 acetone-resin for 5 min at 200W-vacuum 20"Hg, followed by
138 100% resin 4 times at 200W for 5 minutes-vacuum 20"Hg. The resin was removed, and sample
139 pellet was transferred to BEEM conical-tip capsule and filled with 100% fresh modified Spurr's
140 resin. Sample was left to reach bottom of capsule and subsequently left to polymerize for 48 hours
141 in a 65˚C oven followed by 24 hours at room temperature. Ultrathin section ~100nm (silver-gold
142 color) were obtained using Leica UC7 Ultramicrotome and placed on glow-discharged carbon
143 coated 300-mesh Cu grids. Grids were double lead stained with 2% uranyl acetate for 5 minutes
144 and washed with filter sterilized (0.2uM filter) distilled water followed by 5 minute staining with
145 Reynold's lead citrate and subsequent washing as described. All ultrathin TEM sections were
146 imaged on a JEOL 1200 EX TEM (JEOL, Ltd.) at 100 kV, and images were recorded on an SIA-
147 15C charge-coupled device (CCD) (Scientific Instruments and Applications) camera at the
148 resolution of 2,721 by 3,233 pixels using MaxImDL software (Diffraction Limited). All equipment
149 used is located at the Texas A&M University Microscopy and Imaging Center Core Facility
150 (RRID: SCR_022128).
151
152 ImageJ was used to measure all samples in nanometers. Initially, the spore length and core width
153 was measured three times and the average was calculated. Cortex width was measured once from
154 inner membrane to cortex. Statistical analysis using ROUT to remove outliers and data was

155 analyzed using one way analysis of variance (ANOVA) with Tukey test for multiple comparison.
156 Statistical cutoff for significance with a P-value <0.05 was used.
157
158 **Western Blot Analysis**
159 Protein samples were resuspended in 2x SDS-PAGE loading buffer (BioRad) with 5% b-
160 mercaptoethanol and boiled for 10 minutes. Samples were run in a 12% acrylamide SDS-PAGE
161 gels. Proteins were transferred to nitrocellulose membrane and blocked at 4˚C overnight in 3%
162 bovine serum albumin (BSA) in Tris-buffer saline (TBS) (pH7.4) with 0.1% TWEEN20 (TBS-T).
163 Western blots probed with 1:10,000 of primary antibody of mouse anti-CdeC anti-CdeM, and a
164 1:15,000 rabbit anti-SleC in 1% BSA in TBS-T for 1h at room temperature [31] . SleC was a gift
165 from Dr. Joseph Sorg at Texas A&M University. Membrane was washed 3 times for 5 minutes
166 with TBS-T and incubated in secondary antibody 1:10,000 dilution goat anti-mouse HRP (Sigma)
167 in 1% BSA in TBS-T.
168
169 **Antibody Preparation**
170 *E. coli* BL21 (DE3) pRIL was used to overexpress CdeC and CdeM proteins. CdeC and CdeM
171 were respectively overexpressed using pETM11 vector with a C-terminal 6xHIS tag. Plasmids
172 were transformed into BL21 (DE3) pRIL cells and inoculated in 5 mL of Leuria Bertani (LB) broth
173 supplemented with 50 ug/mL chloramphenicol, 10 ug/mL tetracycline, and 50 ug/mL kanamycin
174 in a shaker overnight at 37˚C. Overnight culture was used to inoculate a 300 mL flask with the
175 necessary antibiotics at a 1:100 ratio and grown at 37˚C until OD_{600} reached 0.7-0.8. Fresh LB
176 with the appropriate antibiotics was added until flask reached 1L volume. Once OD_{600} was reached
177 culture was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 18 hrs at

178 21˚C. Cultures were pelleted down at 6500 rpm for 10 minutes and stored at -80˚C. Pellet was
179 resuspended in 5mL of soluble buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1mM
180 phenylmethanesulfonyl fluoride (PMSF), pH=8) and sonicated 15 seconds on, 15 seconds on ice
181 for a total of 6 cycles at 50% amplitude. Lysate was pelleted down at 6500 rpm at 4˚C for 30
182 minutes and subsequently filtered with a 0.22 uM filter.
183
184 Purification of recombinant CdeC and CdeM was done using Akta Start. Briefly, filtered soluble
185 protein extract were loaded on HisTrap FF crude column (GE Healthcare), washed with 15 column
186 volumes (CV) of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH=8).
187 Proteins were eluted with 10 CV of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM
188 imidazole, pH=8). Samples were eluted as a single peak and subsequent fractions were analyzed
189 by 15% SDS-PAGE and stained with Coomassie G250 to determine purity of fractions. To
190 quantify protein Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific) was used.
191
192 5 BALB/c mice, 7-9 weeks old were used for immunization with CdeC and CdeM respectively. A
193 pre-immunized bleed was taken at day -1 from isofluorene anesthetized mice. Blood was incubated
194 for 30 minutes at room temperature to coagulate and then centrifuged at 5000 rpm for 10 minutes
195 at 4˚C. Serum was taken and stored in a new tube. At day 0, 20 ug of recombinant protein was
196 emulsified with Freund's Complete Adjuvant (FCA) in a 1:1 ratio. Mice were anaesthetized and
197 injected with 200 uL of protein emulsified in FCA subcutaneously in the nape. Mice were
198 inoculated as described at day 14 and 28 however using Freund's Incomplete Adjuvant (FIA). At
199 day 42, mice were anaesthetized with 5% isoflurane and maintained at 1.5% and a cardiac puncture
200 was performed to collect blood. Serum was collected as previously described. Animal protocol

201 was approved by the Comité de Bioética of the Facultad de Ciencias Biológicas at the Universidad

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203

204 **Results and discussions**

205

206 **Experimental design of spore coat and exosporium extraction.**

207 Two chemical methods to extract the spore coat and exosporium proteins have been extensively
208 utilized in *C. difficile* spore-research [10, 15-23]. However, their extraction efficiency of these two
209 outermost layers remains unclear. To compare treatments, initially as shown in Figure 1, spores
210 were treated with either EBB or USD. EBB treated samples were boiled 10 minutes and then spun
211 down, supernatant containing spore coat and exosporium extracts was saved and remaining spore
212 pellet was washed to remove residual extraction buffer (Fig. 1), a second and third subsequent
213 extraction were performed to remove and identify any residual protein not removed with a single
214 extraction. In the case of USD, spores were incubated at 37˚C for 90 minutes and after incubation
215 spun down (Fig. 1); Supernatant was saved, and spore pellet was washed. A second and third
216 subsequent extraction were performed as described. To analyze proteins within the core and cortex,
217 spores treated with USD or EBB, 1˚, were incubated with 10 mg/mL of lysozyme overnight. The
218 treatment with lysozyme would allow releasee of proteins within the cortex and core that were not
219 able to be extracted with EBB or USD alone. For all treatments, exosporium/spore coat extracts
220 and treated spores were run through SDS PAGE and immunoblotted for western blot analysis (Fig.
221 1).

222

223 **Transmission Electron Microscopy of Treated Spores and Measurements**

224 To visualize the efficiency of extraction of the spore surface layers 1˚ treated spores with USD,
225 and EBB were imaged through TEM. As a controls, untreated and Laemmli-treated spores were
226 also included. As seen in Figure 2, wild type treated spores have electron dense outer layer, the

227 exosporium, the classical electron-dense bumps shown in thick-exosporium spores, and hair-like
228 projections, followed by the underlying spore coat, cortex, and core (Fig. 2A). However, a single
229 EBB- or USD-treatment was able to remove the majority of the spore coat and exosporium layers,
230 removes the majority of the outermost outermost layers, leaving the spore-peptidoglycan cortex
231 exposed (Fig. 2A). Electron micrographs of Laemmli-treated spores had a thin layer of electron
232 dense material in the outermost area of the spore, suggesting that significant spore coat/exosporium
233 residual material remains in the spore surface (Fig. 2A). Altogether, these results demonstrate that,
234 while all three treatment remove the spore coat and exosporium layers, EBB and USD are more
235 efficient.
236
237 To quantitatively assess the impact of these chemical treatments on the ultrastructure of the spore
238 layers, the length of the entire spore, cortex and spore core where measured. The spore length
239 significantly decreased by ~40 % in spores treated with Laemmli, USD, and EBB when compared
240 to untreated spores (Fig. 2B). *C. difficile* R20291 spores had an average length of ~ 1,000 nm
241 whereas on average Laemmli-, USD-, and EBB-treated spores were ~ 600 nm in length (Fig. 2B).
242 Comparing effect of treatment on the spore core indicated a decrease in core diameter in all the
243 treated samples, however this was not statistically significant. (Fig 2C). There was a ~20%
244 reduction in core diameter in EBB treated spores compared to untreated (Fig. 2C). The average
245 diameter of the core in untreated spores was 455 nm compared to 363 nm in EBB treated spores
246 (Fig. 2C). When comparing the cortex there was significant decrease in cortex between Laemmli
247 and EBB, and Laemmli and USD treated samples (Fig 2C). The decreased cortex width of EBB
248 and Laemmli treated spores could be potentially due to boiling step during extraction leading to
249 significant shrinkage in width, step that is absent in USD-treated spores (Fig. 1). No significant

250 differences where observed in spore core width between untreated and Laemmli-treated spores,
251 and between USD- and EBB-treated spores (Fig. 2D). Collectively, these results demonstrate that
252 while all three treatments (i.e., Laemmli, USD and EBB) remove the spore coat and exosporium
253 layer, those with a boiling step (i.e., Laemmli and EBB) affect the width of the spore cortex, but
254 not spore core.
255
256 **Impact of EBB and USD-treatments in spore coat and exosporium extracts.**
257 Having demonstrated that all three chemical treatments remove most of the spore surface layers,
258 we thought to correlate the ultrastructure of spores treated with EBB, USD or Laemmli with the
259 efficiency of removal of these spore surface proteins by analyzing their protein-profile by SDS-
260 PAGE. For this we treated the spores with EBB, USD or Laemmli, spun down and the spore-pellet
261 and supernatant electrophoresed in an SDS-PAGE (Fig. 1 and 3A,B). Notably, a spore coat and
262 exosporium EBB extracts from a single EBB-treatment resolved a significantly more protein than
263 Laemmli-extracted spore coat and exosporium proteins (Fig. 1 and 3A). Indeed, comparison of the
264 remaining pellet of EBB- and Laemmli-treated spores shows that EBB-treated spores have less
265 remaining spore coat / exosporium material attached (Fig. 1 and 3A). A second (2º) and third (3º)
266 EBB treatment led to complete removal of the spore coat and exosporium extracts and spore-
267 pellets containing negligible remnants of spore coat and exosporium material (Fig. 1 and 3A). In
268 contrast to EBB, although a first USD-treatment yielded substantial protein levels of spore coat
269 extracts, several high and low-molecular weight protein species remained in USD-spore pellets.
270 Indeed, a second USD-treatment led to significant spore coat and exosporium extracts being
271 removed of USD-treated spores (Fig. 3B). No additional protein was removed after a third USD
272 extraction (Fig. 3B). However, spore pellets from a second and third treatment with USD still

273 retained detectable levels of low molecular mass protein species (~10 to 15 kDa) (Fig. 3B).
274 Collectively, these results indicate that EBB is more effective in spore coat and exosporium
275 extraction from *C. difficile* spores than USD and Laemmli.
276
277 **Impact of chemical treatments in removal of exosporium and spore coat protein markers.**
278 While the above electrophoresed protein profiles correspond to total spore coat and exosporium
279 extracts, the removal efficiency of each of these layers is unclear. Therefore, we utilized antibodies
280 against three proteisn as markers. For the spore coat marker, we selected the cortex-lytic enzyme
281 SleC, known to be located in the interface between the spore cortex [32]. For exosporium markers,
282 we selected the cysteine-rich and morphogenetic proteins, CdeC and CdeM [15, 17, 29]; where, CdeM
283 is located on the outermost layer of the exosporium whereas CdeC is found within the exosporium
284 with presumable interactions with the spore coat [17, 33].
285
286 For this, first, aliquots of the 1st EBB extraction blotted against anti-CdeM shows immunoreactive
287 bands at ~70 kDa, ~55 kDa, and ~ 27 kDa (Fig. 3C). In the case of subsequent EBB extractions
288 (2˚ and 3˚), there were no immunoreactive bands in the extracted supernatant as well as in the
289 spore pellet, indicating that the exosporium layer was completely removed (Fig. 3C). Again,
290 Laemmli-treated spores were used as a control. Supernatant of Laemmli-treated spores containing
291 spore coat and exosporium extracts show the presence of ~ 70 and 27 kDa immunoreactive species
292 (Fig. 3C), while Laemmli spore-pellet completely lacked CdeM immunoreactive bands (Fig. 3C).
293 Immunoblotting using anti-CdeM antibodies of 1st USD spore extracts display immunoreactive
294 bands at ~ 70 kDa and 55 kDa, however no 27 kDa immunoreactive band was detectable, and no
295 subsequent CdeM-specific immunoreactive band was observed in subsequent USD spore coat /

296 exosporium extracts (Fig 3D). Moreover, no remnants of CdeM were detected in the USD-treated
297 spore pellet (Fig. 3D). These results demonstrate that all three treatments efficiently remove the
298 exosporium layer but differ in their levels of dissociation of the various CdeM oligomeric species
299 present in the spore coat / exosporium extracts.
300
301 Next, to explore whether CdeC, which is an exosporium protein suggested to be at the interface of
302 the spore coat and the exosporium electron dense layer[10, 17], was removed as efficient as CdeM,
303 immunoblots where analyzed against anti-CdeC antibodies. Results demonstrate that a single
304 treatment of EBB or Laemmli was sufficient to extract all immunodetectable CdeC, with no CdeC
305 remnants in spore pellets (Fig. 3E,F). In the case of USD-treatment, immunoreactive bands were
306 observed at ~130, 70 and and 55 kDa, however residual ~55 kDa immunoractive band was detected
307 in USD-treated spores, which was successfully removed after a second treatment with USD (Fig.
308 3F). These results demonstrate that EBB and Laemmli are more efficient than USD at extracting
309 proteins that are at the interface with the spore coat, such as CdeC.
310
311 Finally, the a spore coat / peptidoglycan interface marker protein, SleC, was used to detect efficient
312 extraction of spore surface layers as well as cortex proteins[31]. During sporulation SleC is processed
313 and appears as three immunoreactive bands in spore coat / exosporium extracts, where full length
314 ~55 kDa, pro-SleC ~37 kDa, and active SleC ~27 kDa[32]. An initial EBB extraction displayed
315 immunoreactive bands for all three versions of SleC at the expected ~55 kDa, ~37 kDa, and ~27
316 kDa, while EBB-treated spore pellets had a very faint band at ~37 kDa (Fig 3G), suggesting the
317 presence of remnants at the interface of the spore peptidoglycan / spore coat layers or remnants of
318 the spore coats themselves. A second EBB-treatment was sufficient to completely remove SleC

319 remnants (Fig. 3G). Notably, Laemmli gave consistent immunoreactive bands for all forms of SleC
320 but failed to completely remove SleC as seen with a strong immunoreactive band within the treated
321 spore at ~37 kDa (Fig 3G, 3H). In the case of USD, an initial USD-treatment extracted the majority
322 of SleC, with a faint 37 kDa immunoreactive present in the spore pellets, which was also present
323 upon treating USD-treated spores with a second USD-treatment and completely removed SleC
324 from the spore pellet fraction (Fig 3H). These observations indicate that a single EBB-treatment is
325 sufficient to remove the majority of SleC from the spore coat, and very likely other spore coat
326 proteins.
327
328 **Impact of EBB and USD in lysozyme-triggered germination**
329 The spore coat layer is known to act as an impermeable barrier to molecules > 5 kDa, such as
330 enzymes, including proteinase K and lysozyme [30, 34, 35]. Moreover, the spore peptidoglycan cortex
331 in decoated spores is susceptible to lysozyme degradation, artificially triggering spore germination
332 and outgrowth [30]. We reasoned that depending on the efficiency with which EBB and USD
333 removed the spore coat, EBB and USD-treated spores will have different levels of susceptibility
334 to lysozyme-germination. Phase contrast analysis of untreated, EBB-, or USD-treated spores
335 reveals that untreated and USD-treated spores are phase bright (Fig. 4A,B), consistent with them
336 remaining dormant. By contrast, the majority (~90 %) of EBB-treated spores become phase grey
337 (Fig. 4A,B), which could be attributed to the boiling step of *C. difficile* spores, which is known to
338 release spore core DPA contents in *C. difficile* and other spore-formers [17, 36].
339
340 To assess effects of USD and EBB treatment on spore coat permeability and extraction efficiency,
341 spores were treated with lysozyme (10 mg/mL) overnight at 37 ˚C for 24 hours [17, 23]. Untreated

342 spores appeared phase bright, which is indicative that lysozyme was unable to trigger germination.
343 By contrast, most of the EBB and USD treated spores became phase dark which indicates that
344 lysozyme degraded the cortex, leading to spore core hydration (Fig. 4A,B). However, a subfraction
345 of USD-treated spores subsequently treated with lysozyme appeared phase grey. This suggests that
346 unlike phase dark spores, phase grey spores either partially released their spore core DPA and/or
347 exhibit partial spore core rehydration. Alternatively, it is likely that phase grey spores retain
348 remnants of the spore coat which impedes lysozyme-germination and subsequent spore core
349 hydration. Collectively, these observations demonstrate that EBB-treatment provides a
350 homogenous population of decoated spores that can be utilized in downstream applications.
351
352 **Impact of lysozyme digestion in the protein profile of EBB- and USD-treated spores.**
353 The results showing that EBB and USD remove most of the spore coat and that these decoated
354 spores are readily digested with lysozyme led us to speculate whether these steps could be utilized
355 to pop-open the spore core for electrophoresis. As observed, resuspending EBB-, USD- or
356 Laemmli-treated spores in Laemmli sample buffer followed by 5 min incubation at 90 ºC prior to
357 electrophoresing them in an SDS-PAGE gel (Fig. 5), did not release the proteins contained in the
358 spore peptidoglycan cortex and in the spore core, demonstrating the resilience of decoated spores
359 to harsh treatments. However, upon incubating EBB- or USD-decoated spores with lysozyme for
360 24 hrs at 37 ˚C and subsequently electrophoresed, we observed an increase in the levels of proteins
361 being electrophoresed (Fig. 5), which improved further when incorporating Laemmli buffer and a
362 5 min incubation at 90 ºC prior to electrophoresis (Fig. 5). Notably, despite Laemmli buffer being
363 able to remove the spore coats (Fig. 2 and 3), Laemmli-decoated spores subsequently treated with
364 lysozyme did not release spore core content material. In summary, these results evidence that

365 electrophoresing lysozyme-treated decoated spores provides a method to release the spore
366 peptidoglycan cortex and spore core proteins.
367
368 **Conclusions.**
369 This work provides evidence that EBB-decoating treatment is more efficient than USD in
370 removing the spore coat and exosporium layers. Additionally, results also demonstrate that EBB-
371 treated spores can be digested with lysozyme to release the spore peptidoglycan cortex and spore
372 core proteins. These techniques can be adapted in methods seeking to fractionate the different
373 layers of the spore (i.e., exosporium, spore coat, spore peptidoglycan cortex, and spore core).
374
375
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515

516 **Figure legends**

517 **Figure 1. Schematic of extraction treatment.** Purified R20291 *C. difficile* spore ($5x10^9$
518 spores/mL) were pelleted down and resuspended in USD or EBB. USD treated spores were
519 incubated at 37˚C for 90' min. EBB treated spores were boiled for 10' min. Spores were pelleted
520 down for 5'min at 14,000 rpm and supernatant 1˚S saved for SDS-PAGE and Western Blot
521 Analysis. Spore pellet (1˚) was washed 3x with distilled H_2O and centrifuged in between washes
522 at 14,000rpm for 5'min. 1˚ treated spores were subsequently treated two more times with USD or
523 EBB as described above denoted as 2˚ and 3˚. Supernatant and a portion of spore pellet is saved
524 between each step for analysis. 1˚ spores were treated with 10mg/mL for 24 hours at 37˚C to
525 identify effects of treatments on spore coat integrity.

526

527 **Figure 2. Transmission Electron Microscopy of Treated *C. difficile* spores**

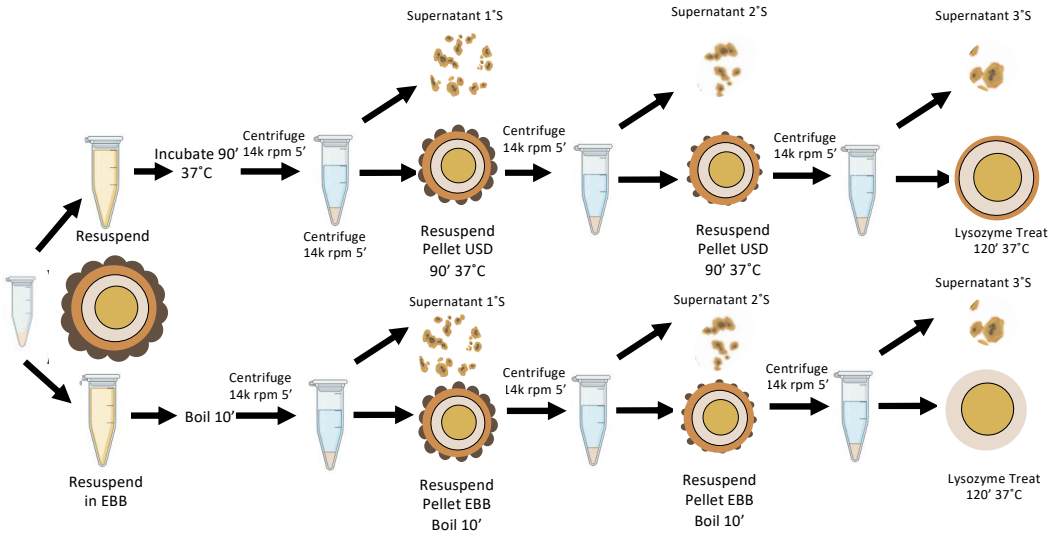
528 A) Transmission Electron Microscopy images of Wild Type, EBB, USD, and Laemmli treated *C.*
529 *difficile* spores. Measurements were taken using ImageJ of spore diameter, core width, and core
530 diameter (B to D) 9 wild type spores and 10 of each treated spore were measured. Statistical
531 significance was performed using a 1 way ANOVA with Tukey's multiple comparisons test (not
532 significant-ns, * $p < 0.05$; ** $p < 0.01$, *** $p<0.001$, **** $p<0.00001$).

533

534

535 **Figure 3. Extraction of Spore Outer layers using EBB and USD. A)** 15% SDS-PAGE of
536 purified R20291 spores treated with USD, EBB, and 2x Laemmli. Spores were treated as described
537 in Methods and Figure 1**.** 1˚,2˚, 3˚ indicate consecutive extraction treatment of spores, Laemmli 1˚

538 indicates one time Laemmli extraction. Western blot analysis of treated samples using: **B)** anti-
539 CdeM antibodies, arrows indicate immunoreactive bands at ~70 kDa, 55 kDa, and 27 kDa
540 respectively. **C)** anti-CdeC antibodies, arrows indicate immunoreactive bands indicating extraction
541 of CdeC with bands at ~130 kDa, 55 kDa, and 37 kDa, respectively. **D)** anti-SleC antibodies
542 indicate immunoreactive bands at 55kDa full length SleC, 37 kDa pro-SleC, and 27kDa active
543 SleC. Higher olimeric bands likely oligomerization of the protein.
544
545 **Figure 4. Effect of extraction on spore coat permeability to lysozyme.** Effects of USD and EBB
546 treatment on spore coat integrity and lysozyme resistance. R20291 *C. difficile* spores were treated
547 for 90 min at 37˚C incubator with USD, washed and imaged under phase contrast. EBB treated
548 spores were boiled for 10 min, centrifuged for 5 min at 14,000 rpm and washed. Spores were
549 treated with 10 mg/mL lysozyme for 24 hours at 37˚C. Effects on spore coat integrity and
550 degradation of peptidoglycan indicated by phase dark spores under phase contrast. B) Lysozyme
551 treated spores were quantified and percent phase bright, grey, and dark were graphed.
552
553 **Figure 5. Lysozyme treatment of decoated-spores.** 15% SDS-PAGE of purified R20291 spores
554 treated with USD, EBB, and 2x Laemmli. Spores were treated as described in Methods and Figure
555 1. 1˚ and 2˚ spores treated with 10mg/mL lysozyme, 1˚spores were not treated with lysozyme after
556 initial USD/EBB treatment. 1˚Laemmli treated spores with and without lysozyme also shown.
557 ~14kDa band on SDS corresponding to lysozyme indicated.
558



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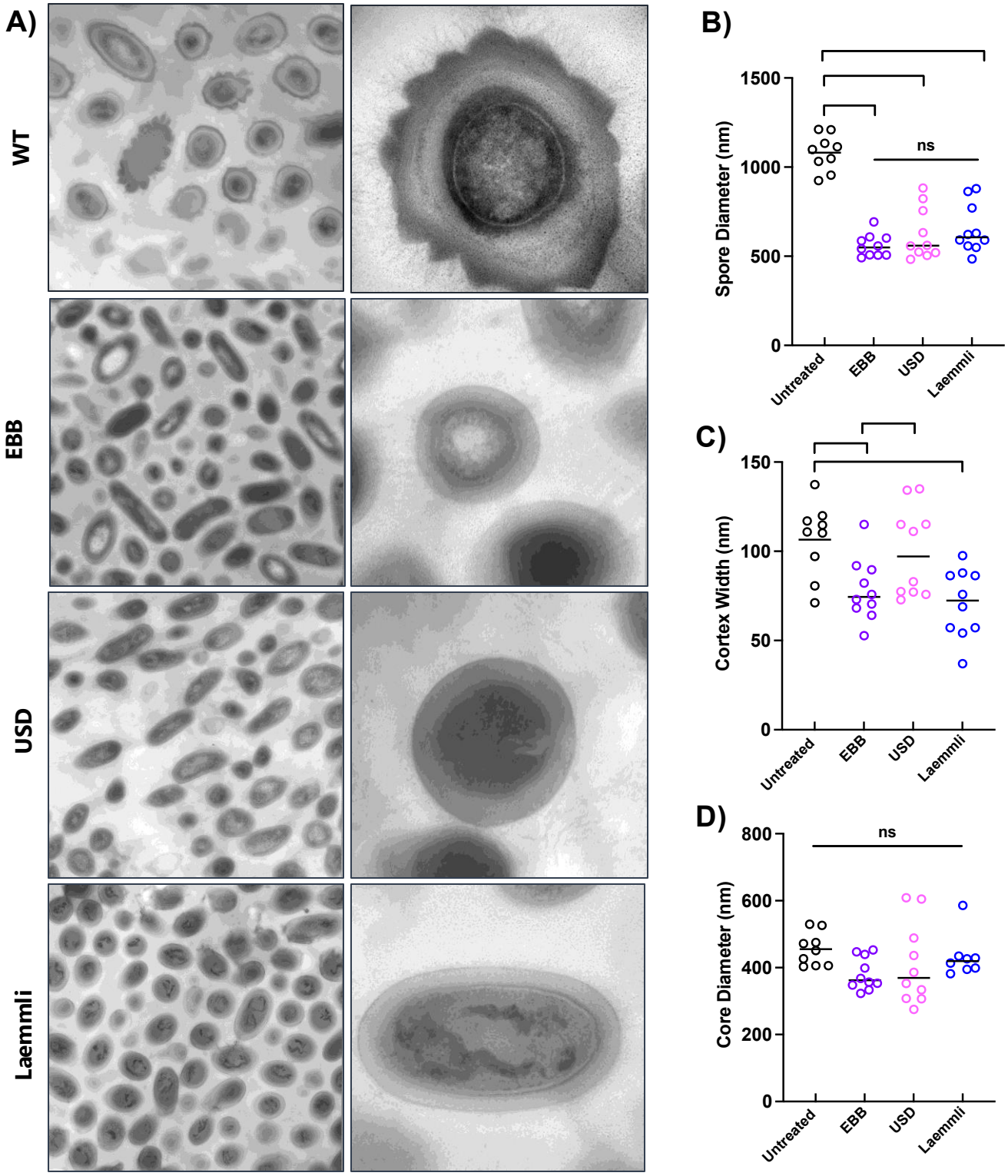
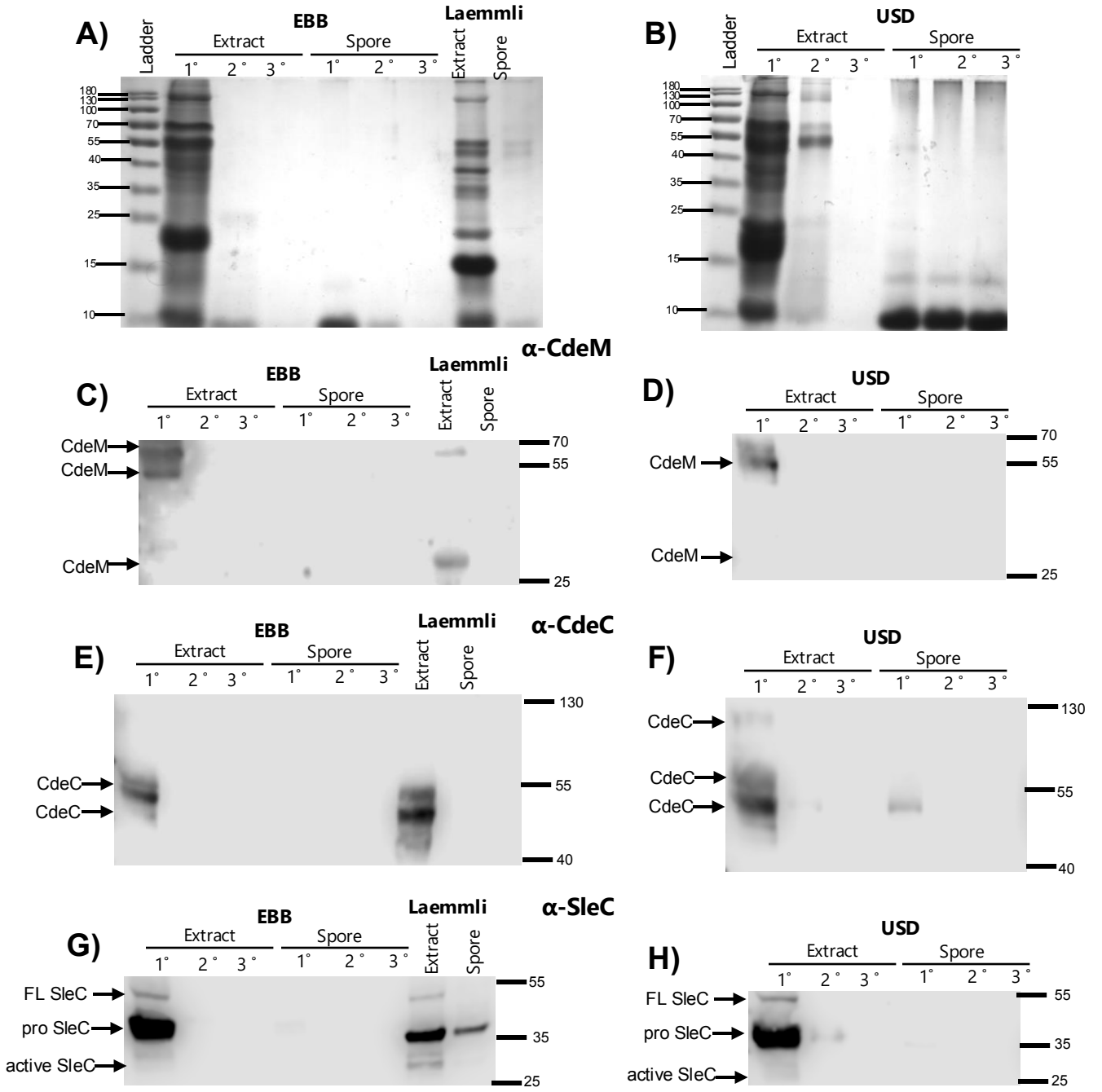


Figure 2. Transmission Electron Microscopy of Treated *C. difficile* spores A) Transmission Electron Microscopy images of Wild Type, EBB, USD, and Laemmli treated *C. difficile* spores. Measurements were taken using ImageJ of spore diameter, core width, and core diameter (B to D) 9 wild type spores and 10 of each treated spore were measured. Statistical significance was performed using a 1 way ANOVA with Tukey's multiple comparisons test (not significant-ns, * $p < 0.05$; ** $p < 0.01$, *** $p<0.001$, **** $p<0.00001$).



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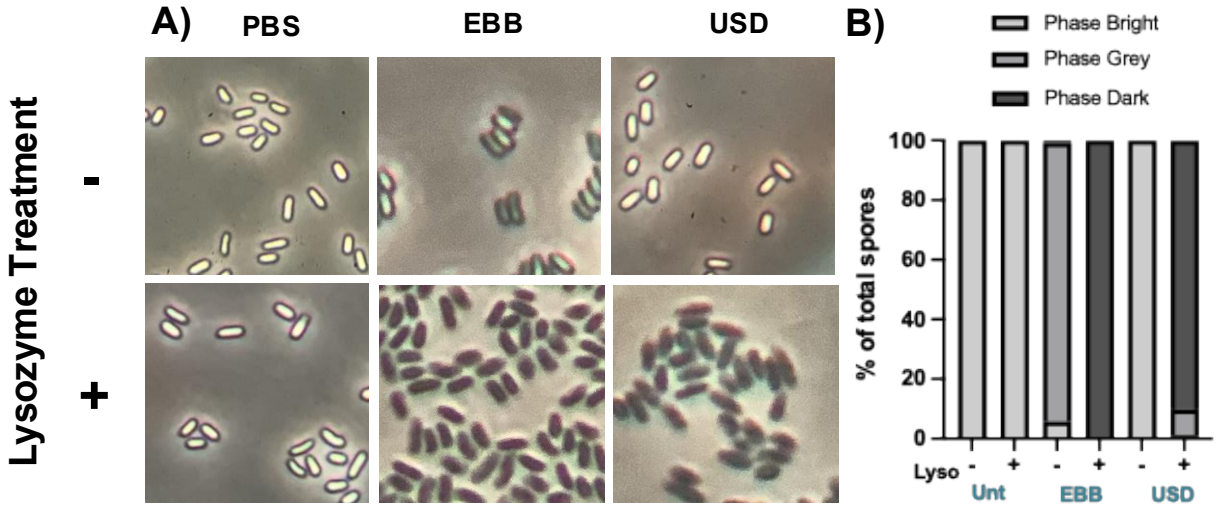

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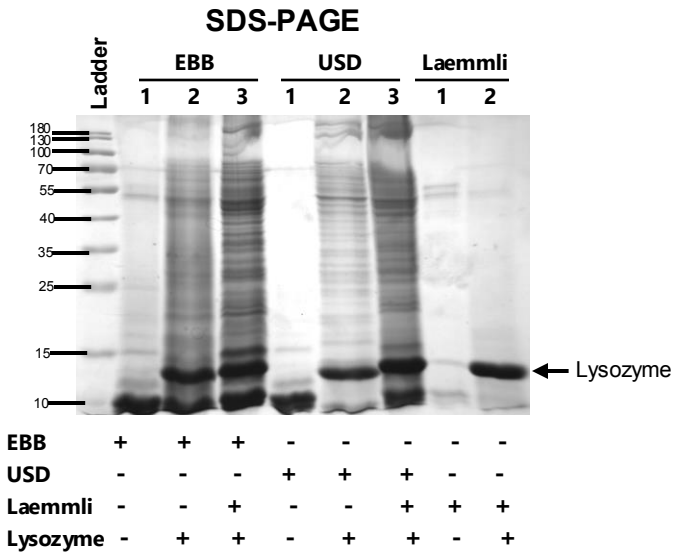

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