Optimization of Spore Coat and Exosporium extracts removal from *Clostridioides difficile* Spores.

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Abstract

Analysis of the protein composition that make up *C. difficile* spores is essential in understanding the protein composition of its surface layers however the spore surface is composed of protein complexes hindering protein analysis. Here we compare commonly used protein extraction methods used to extract proteins of *C. difficile* spores. Our results provide a systematic analysis of how two commonly used spore coat / exosporium extraction methods compare in efficiency of protein removal and spore structural analysis.

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 essential for disease manifestation[4], the production of *C. difficile* spores during disease is essential for disease 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, and while the exosporium layer can be easily removed by protease digestions (i.e., trypsin or proteinase-k) while the spore coats remain intact[17]. Despite the easiness to proteolytically remove the exosporium layer, it is unclear whether the spore coat and exosporium layer are efficiently removed through chemical methods, commonly employed in *C. difficile* spore-research. Therefore,

56 in this work, our aim is to provide a systematic analysis of how two chemical methods (i.e., USD
57 and EBB) that are commonly employed to remove the spore coat /exosporium extracts for
58 immunoblotting analysis, remove these layers and impact the functional aspects of *C. difficile*
59 spores. For this, we utilized two exosporium markers (i.e, CdeC and CdeM)[10, 18, 19], and the
60 peptidoglycan-spore coat interface specific spore cortex lytic enzyme SleC[20, 21], to monitor the
61 efficiency of extraction of these layers through immunoblotting and transmission electron
62 microscopy.
63
64 **Methods**
65 **Spore Preparation and Purification.**
66 *C. difficile* strain R20291 was grown in Brain Heart Infusion broth (BHIS) (Difco) supplemented
67 with 0.5% yeast extract and 0.1% cysteine anaerobically at 37˚C. Overnight cultures were grown
68 in BHIS broth with 0.1% sodium taurocholate and 0.2% d-fructose. Cultures were diluted to an
69 OD_{600} of 0.5 and 250 uL was plated on 70:30 sporulation media under anaerobic conditions for 5
70 days at 37˚C. Sporulating cultures were collected from plates, resuspended in ice-cold distilled
71 water, and left in 4˚C overnight to allow for lysis of vegetative cells. After incubation, cells were
72 centrifuged and pellets washed with ice-cold distilled water 5 times followed by repeated
73 centrifugation and resuspension to remove vegetative cell debris. Spores were separated using a
74 60% sucrose gradient and centrifuged for 20 min at 14,000 rpm spores were then washed with ice-
75 cold water to remove residual sucrose. Washing and sucrose gradient repeated until spores reached
76 99% purity. Once pure, spores were quantified using a NeuBauer chamber and stored at -80˚C
77 until use at a $5x10^9$ spores/mL concentration.
78

79

80 **Spore Extraction Methods**

81 1.5x10^7 spores pelleted down and resuspended in 50 uL of buffer. Samples with EBB (EBB 8M
82 Urea, 2M Thiourea, 4% w/v SDS, 2% w/v β-mercaptoethanol) were boiled 10 minutes and spun
83 down at 15k rpm for 5 minutes. Supernatant containing spore extract was saved and spore pellet
84 washed three times to remove residual spore extract. Spore was then retreated with EBB for a total
85 of three extraction with supernatant saved between each step. USD (8M Urea, 1% w/v SDS, 50
86 mM DTT, 50 mM Tris-HCl (pH 8)samples were treated for 90 minutes at 37˚C and pelleted down.
87 Supernatant was saved and spore pellet washed. 1˚ USD or 1˚ treated spores were incubated with
88 lysozyme for 37˚C overnight for 24 hours.

89

90

91 **Lysozyme Treatment**

92 *C. difficile* spores were treated with lysozyme to analyze the effects of the extraction buffers on
93 the integrity of the spore coat. Spores ($1x10^7$ spores) were treated with 10mg/mL lysozyme in 25
94 mM phosphate buffer (pH 7.4) at 37˚C for 2 hours. Samples were pelleted and washed with
95 distilled water and analyzed under phase contrast microscopy.

96

97

98 **Transmission Electron Microscopy**

99 For transmission electron microscopy, *C. difficile* spores (10^{10}) were fixed overnight with 3%
100 glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2) at 4˚C. Samples were centrifuged at 14k rpm for
101 5 minutes and supernatant discarded. They were then stained with1% osmium tetroxide in 0.05M

102 HEPES buffer (pH 7.4) overnight at 4˚C. Treated samples were washed 5x with distilled water.
103 The spores were dehydrated stepwise in 30%, 50%, 70%, 90% for 15 minutes respectively,
104 followed by dehydration with 100% acetone 3 times for 30 minutes at each step. A small amount
105 of acetone is left covering the sample to prevent rehydration. Spore samples were embedded in
106 modified Spurr's resin (Quetol ERL 4221 resin; EMS; RT 14300) in a Pelco Biowave processor
107 (Ted Pella, Inc.). Initially, 1:1 acetone-resin for 10 min at 200W-with no vacuum, 1:1 acetone-
108 resin for 5 min at 200W-vacuum 20"Hg, followed by 100% resin 4 times at 200W for 5 minutes-
109 vacuum 20"Hg. The resin was removed, and sample pellet was transferred to BEEM conical-tip
110 capsule and filled with 100% fresh modified Spurr's resin. Sample was left to reach bottom of
111 capsule and subsequently left to polymerize for 48 hours in a 65˚C oven followed by 24 hours at
112 room temperature. Ultrathin section ~100nm (silver-gold color) were obtained using Leica UC7
113 Ultramicrotome and placed on glow-discharged carbon coated 300-mesh Cu grids. Grids were
114 double lead stained with 2% uranyl acetate for 5 minutes and washed with filter sterilized (0.2uM
115 filter) distilled water followed by 5 minute staining with Reynold's lead citrate and subsequent
116 washing as described. All ultrathin TEM sections were imaged on a JEOL 1200 EX TEM (JEOL,
117 Ltd.) at 100 kV, and images were recorded on an SIA-15C charge-coupled device (CCD)
118 (Scientific Instruments and Applications) camera at the resolution of 2,721 by 3,233 pixels using
119 MaxImDL software (Diffraction Limited). All equipment used is located at the Texas A and M
120 University Microscopy and Imaging Center Core Facility (RRID: SCR_022128).
121

122 **Western Blot Analysis**

123 Protein samples were resuspended in 2x SDS-PAGE loading buffer (BioRad) with 5% b-
124 mercaptoethanol and boiled for 10 minutes. Samples were run in a 12% acrylamide SDS-PAGE

125 gels. Proteins were transferred to nitrocellulose membrane and blocked at 4˚C overnight in 3%
126 bovine serum albumin (BSA) in Tris-buffer saline (TBS) (pH7.4) with 0.1% TWEEN20 (TBS-T).
127 Western blots probed with 1:10,000 of primary antibody of mouse anti-CdeC anti-CdeM, and anti-
128 SleC in 1% BSA in TTBS for 1h at room temperature. Membrane was washed 3 times for 5
129 minutes with TBS-T and incubated in secondary antibody 1:10,000 dilution goat anti-mouse HRP
130 (Sigma).

131 **Results and discussions**

132

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

134 To test efficiency of extractions we used three spore markers. CdeC and CdeM for efficiency of
135 exosporium extraction since they have been identified as key morphogenic exosporium proteins.
136 For efficient extraction of spore cortex constituents SleC was used as a marker. Initially as shown
137 in Figure 1, spores were treated with either EBB or USD. EBB treated samples were boiled 10
138 minutes and then spun down, supernatant containing spore surface layer extracts was saved and
139 remaining spore pellet was washed to remove residual extraction buffer. USD treated spores were
140 incubated at 37˚C for 90 minutes and after incubation spun down. Supernatant was saved and spore
141 pellet was washed. Initial spores treated with USD or EBB were then treated with 10 mg/mL of
142 lysozyme which would degrade cortex. For all treatments extracts of treated spores were run
143 through SDS PAGE and immunoblotted for western blot analysis.

144

145

146 **Impact of EBB and USD in spore coat and exosporium extracts.**

147 Efficiency of extractions using EBB and USD were tested through SDS-PAGE and western blot
148 analysis. Extracts of initial EBB treated spores (1˚) indicate a substantial amount of protein is
149 removed from the spore. A variety of proteins are extracted as seen through the SDS-PAGE protein
150 banding. A second subsequent EBB extraction (2˚) extracts residual protein likely not extracted
151 from the initial treatment (1˚). Protein bands are evident at ~10, ~15, and ~25 kDa. A third EBB
152 extraction (3˚) most of the surface proteins are extracted, no protein is seen in SDS-PAGE stained
153 with Coomassie blue. The spores were run through SDS-PAGE to identify protein left on spore

154 surface after each treatment. Initial 1˚ spores have protein present within the SDS-PAGE indicating
155 not all protein was removed with the initial extraction from the spore surface consistent with the
156 protein that was able to be extracted following the 2˚ EBB extraction. Spores of 2˚ extraction
157 display protein bands indicating some protein was left on spore surface. However, spores of 3˚
158 treatment no protein is present in SDS-PAGE indicating most if not all protein is removed with 2
159 total treatments. Laemmli control efficiently extracts surface proteins of however the amount is
160 less compared to EBB. Similarly, one treatment with Laemmli does not completely remove spore
161 surface proteins as seen through residual protein in the spore.
162 USD spore extracts as seen in Figure 2A reveal a large amount of protein is removed from the
163 spore surface with an initial extraction (1˚). Following a subsequent extraction (2˚) there is
164 additional protein that is able to be removed as seen in the SDS-PAGE. This indicates that not all
165 the protein is able to be removed from an initial treatment and substantial amount is left on the
166 spore surface. Similar to EBB treatment, there is little to no protein seen in spore USD extracts of
167 3˚. Spores treated with USD (1˚) have residual protein on spore surface after initial USD extraction
168 and a protein band is clearly seen ~10 kDA likely whole spore running through SDS PAGE.
169 Residual protein is seen on spore in both 2˚ and 3˚ indicating spore surface still contains protein.
170
171 **Effect of EBB and USD in exosporium markers.**
172 Western blot analysis using anti-CdeM and anti-CdeC antibodies were used as markers for efficient
173 extraction of exosporium proteins. CdeM is located on the outermost layer of the exosporium
174 whereas CdeC is found within the exosporium with interaction with spore coat. EBB extracts of
175 1˚ using anti-CdeM antibodies display immunoreactive bands at ~70 kDa, ~55 kDa, and 27 kDa
176 indicating formation of higher order oligomeric species as well as monomer (~27 kDa) (Figure

177 2B). Immunoblotting using anti-CdeM antibodies of 1˚ USD spore extracts display
178 immunoreactive bands at ~70 kDa and 55 kDa however no monomer is seen ~27kDa. Laemmli
179 extractions display immunoreactive bands at ~70 kDa and ~27 kDa but lacks the 55 kDa band seen
180 within the USD and EBB extracts. This suggest usage of traditional Laemmli may overlook certain
181 oligomeric bands as we've seen in USD and EBB. EBB spore extracts of 2˚ and 3˚ lack
182 immunoreactive bands indicating a majority of CdeM protein is removed with an initial extraction.
183 Likely, bands seen within SDS-PAGE in Figure 2A may be unknown spore surface constituents.
184 No CdeM immunoreactive bands were seen on 1˚ and 2˚ treated EBB spores. In the case of USD,
185 like EBB there were no immunoreactive bands seen in the treated spores as well as the subsequent
186 2˚ and 3˚ extractions indicating a majority of the CdeM protein is efficiently removed with a single
187 extraction. Laemmli is able to efficiently extract CdeM as no protein is seen in 1˚ Laemmli treated
188 spores.
189
190 Use of anti-CdeC antibodies, EBB extracts displayed immunoreactive band at ~55 kDa
191 corresponding to the monomeric form of CdeC (Figure 2C). Although predicted to be ~44.4 kDa
192 CdeC present immunoreactive bands at 55 kDa likely due to interactions with other spore surface
193 constituents and or formation of oligomers. No immunoreactive bands are seen in EBB extracts of
194 2˚ and 3˚ or in extracted spores indicating that EBB efficiently extracted most of CdeC within
195 spore surface. USD spore extracts 1˚, western blot indicated immunoreactive bands at ~55 kDa as
196 well as ~130 kDa indicating formation of oligomers in USD but not EBB. Likely, EBB is able to
197 more efficiently break down CdeC oligomers compared to USD. USD extracts of 2˚ indicate
198 immunoreactive band at 55 kDa suggesting USD failed to remove all CdeC protein from spore
199 surface with an initial extraction. Supporting this, immunoreactive band ~55 kDa is seen in 1˚

200 treated spores again indicating not all protein is removed in an initial extraction and some CdeC
201 remains on spore surface as seen with 2˚ USD extraction removing residual protein that was not
202 removed with the initial extraction. EBB however is able to efficiently remove all CdeC within
203 spore surface whereas USD leaves residual protein and does not efficiently dissociate oligomeric
204 species compared to EBB. Laemmli extraction extracts a majority of CdeC from the exosporium
205 as seen in initial extraction and lack of immunoreactive band in treated spore.
206
207
208 **Effect of EBB and USD in spore coat – peptidoglycan interface marker.**
209 As a peptidoglycan marker SleC was used to detect efficient extraction of spore surface layers as
210 well as cortex proteins. During sporulation SleC is processed and appears as three immunoreactive
211 bands, full length ~55 kDa, pro-SleC ~37 kDa, and active SleC ~27 kDa.Initial EBB extraction 1˚
212 displayed immunoreactive bands for all three versions of SleC at the expected ~55 kDa, ~37 kDa,
213 and ~27 kDa. A second extraction with EBB however had a very faint band indicating most of the
214 protein is extracted with one EBB treatment. Treated EBB spores 1˚ displayed a very faint
215 immunoreactive band suggesting there is some residual SleC not extracted with the initial
216 treatment that is extracted with a second extraction 2˚ as seen in Figure 2D. However, no
217 immunoreactive bands are seen in the treated spores for 2˚ and 3˚. Initial extraction 1˚, with USD
218 extracted a majority of the SleC with expected immunoreactive bands similar to EBB at ~55 kDa,
219 ~37 kDa, and ~27 kDa. However, USD failed to completely extract SleC as immunoreactive band
220 was present with a second USD extraction at ~37 kDa. A weak band is seen ~37 kDa in 1˚ treated
221 spores consistent with USD not completely removing SleC with the initial extraction resulting in
222 immunoreactive bands with a 2˚ extraction. No SleC is extracted with a third extraction or seen in

223 2˚or 3˚ treated spores. This indicates two extractions is sufficient to completely extract SleC from
224 the spore. Laemmli gave consistent immunoreactive bands for all forms of SleC but failed to
225 completely remove SleC as seen with a strong immunoreactive band within the treated spore at
226 ~37 kDa. EBB consistently is able to extract more of the surface layer proteins CdeC and CdeM
227 as well as cortex marker SleC in comparison to USD.
228
229 **Impact of EBB and USD in lysozyme-triggered germination**
230 To assess effects of USD and EBB treatment on spore coat permeability and extraction efficiency,
231 spores were treated with 10mg/mL lysozyme overnight at 37 ˚C for 24 hours. Untreated spores
232 will appear phase bright whereas spores which have degraded cortex due to permeability to
233 lysozyme will appear phase dark. EBB treated spores appear phase bright before treatment
234 however turn phase grey after an initial treatment with EBB treatment likely due to extraction of
235 exosporium and spore coat proteins resulting in rehydration of the core and or DPA loss due to
236 boiling. USD spores in contrast appear phase bright after initial treatment similar to the untreated
237 control suggesting EBB is a harsher treatment compared to EBB. EBB spores were treated with
238 Lysozyme and appeared phase dark after 24 h our incubation at 37˚C indicating that EBB affected
239 spore coat permeability leading got cortex degradation. In contrast, USD spores which appeared
240 phase bright after initial extraction were phase dark after lysozyme treatment. This indicates that
241 USD is able to permeabilize the spore coat. Although USD was not as efficient at extraction of
242 spore surface layers in contrast to EBB is it still able to permeabilize the spore coat as seen through
243 phase dark spores in Figure 3.
244
245 **Lysozyme treatment of EBB- and USD-treated spores.**

246 EBB and USD are efficient at extracting the spore surface layers and permeabilizing the spore
247 coat, increasing its susceptibility to lysozyme turning phase dark as seen in Figure 3. To identify
248 effect of extraction on inner spore coat layers 1˚ treated spores (Lane 1) were incubated with
249 lysozyme for 24 hrs at 37 ˚C and subsequently treated with EBB or USD and run through SDS-
250 PAGE (Lane 2). In Figure 4A, treatment of 1˚ spores with lysozyme followed by EBB or USD
251 released increased extraction of protein from the spore. In contrast Lane 3 which was treated with
252 lysozyme and Laemmli had a extracted more protein than Lane 2. The lysozyme is denoted by an
253 arrow at the predicted ~14 kDa.
254
255 Western blot analysis using anti-CdeM antibodies revealed that use of lysozyme did not lead to
256 any further extraction of CdeM consistent with the data from Figure 2 where most if not all CdeM
257 protein is removed with an initial USD or EBB extraction from the spore surface. In the case of
258 anti-CdeC antibody immunoreactive bands are seen at ~70 kDa and ~55 kDa in USD treated spores
259 with lysozyme. As seen in Figure 2, one USD extraction was not sufficient for complete removal
260 of CdeC from the spore surface. CdeC immunoreactive bands are seen in samples treated with
261 lysozyme Lane 2 and Lane 3 and not Lane 1 indicating that lysozyme allowed for further extraction
262 of CdeC from the spore surface layers. CdeC impacts spore coat assembly likely release when
263 lysozyme degrades the cortex and spore coat is degraded. In the case of SleC a marker for cortex
264 SleC immunoreactive bands are present in EBB treated spores Lane 1, and no immunoreactive in
265 Lane 2 where Lane 1 spores were treated with lysozymes for 24 hrs and underwent EBB treatment.
266 However, lysozyme treated spores with Laemmli were able to extract SleC. In the case of USD,
267 SleC is found within 1˚ USD treated spores Lane 1, however no immunoreactive SleC bands are
268 seen in either lysozyme treatment Lane 2 and Lane 3. Similarly in the case of Laemmli treated

269 spores alone an initial Laemmli treatment removed most if not all SleC from the spore as treatment

270 with lysozyme did not release addition SleC protein.

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272

273

274

275 **Figure legends**

276 **Figure 1.** Schematic of extraction treatment. Purified R20291 C. difficile spore (5x109 spores/mL)
277 were pelleted down and resuspended in USD or EBB. USD treated spores were incubated at 37˚C
278 for 90' min. EBB treated spores were boiled for 10' min. Spores were pelleted down for 5'min at
279 14,000 rpm and supernatant 1˚S saved for SDS-PAGE and Western Blot Analysis. Spore pellet
280 (1˚) was washed 3x with distilled H2O and centrifuged in between washes at 14,000rpm for 5'min.
281 1˚ treated spores were subsequently treated two more times with USD or EBB as described above
282 denoted as 2˚ and 3˚. Supernatant and a portion of spore pellet is saved between each step for
283 analysis. 1˚ spores were treated with 10mg/mL for 24 hours at 37˚C to identify effects of treatments
284 on spore coat integrity.

285

286 **Figure 2. Extraction of Spore Outer layers using EBB and USD. A)** 15% SDS-PAGE of
287 purified R20291 spores treated with USD, EBB, and 2x Laemmli. Spores were treated as described
288 in Methods and Figure 1**.** 1˚,2˚, 3˚ indicate consecutive extraction treatment of spores, Laemmli 1˚
289 indicates one time Laemmli extraction. Western blot analysis of treated samples using: **B)** anti-
290 CdeM antibodies, arrows indicate immunoreactive bands at ~70 kDa, 55 kDa, and 27 kDa
291 respectively. **C)** anti-CdeC antibodies, arrows indicate immunoreactive bands indicating
292 extraction of CdeC with bands at ~130 kDa, 55 kDa, and 37 kDa, respectively. **D)** anti-SleC
293 antibodies indicate immunoreactive bands at 55kDa full length SleC, 37 kDa pro-SleC, and 27kDa
294 active SleC. Higher olimeric bands likely oligomerization of the protein.

295

296 **Figure 3. Effect of extraction on spore coat permeability to lysozyme.** Effects of USD and EBB
297 treatment on spore coat integrity and lysozyme resistance. R20291 *C. difficile* spores were treated

298 for 90 min at 37˚C incubator with USD, washed and imaged under phase contrast. EBB treated
299 spores were boiled for 10 min, centrifuged for 5 min at 14,000rpm and washed. Spores were treated
300 with 10 mg/mL lysozyme for 24 hours at 37˚C. Effects on spore coat integrity and degradation of
301 peptidoglycan indicated by phase dark spores under phase contrast.
302
303 **Figure 4. Lysozyme treatment of surface layer extracted spores. A)** 15% SDS-PAGE of
304 purified R20291 spores treated with USD, EBB, and 2x Laemmli. Spores were treated as described
305 in Methods and Figure 1**.** 1˚ and 2˚ spores treated with 10mg/mL lysozyme, 1˚spores were not
306 treated with lysozyme after initial USD/EBB treatment. 1˚Laemmli treated spores with and
307 without lysozyme also shown. ~14kDa band on SDS corresponding to lysozyme indicated. **B)**
308 Western blot analysis of treated samples using **i)** anti-CdeM antibodies, show a lack of
309 immunoreactive bands at ~70 kDa, 55 kDa, and 27 kDa respectively. **ii)** anti-CdeC antibodies,
310 arrows indicate immunoreactive bands indicating extraction of CdeC with bands at 55 kDa, and
311 37 kDa, respectively. **iii)** anti-SleC antibodies indicate immunoreactive bands at 55kDa full length
312 SleC and 37 kDa pro-SleC.
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Figure 1: Schematic of spore treatment

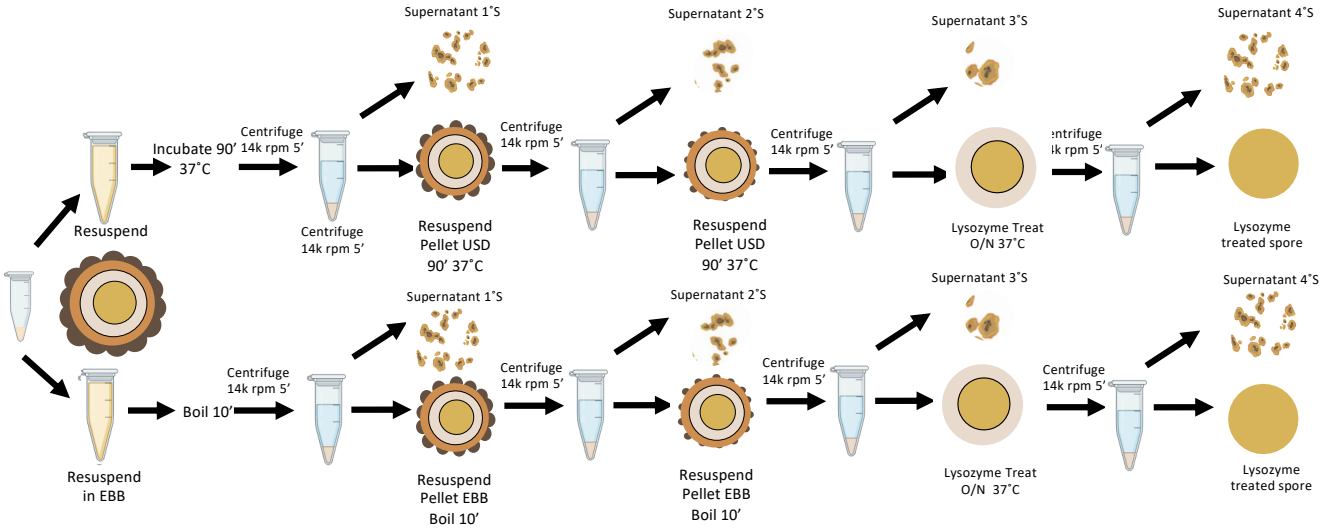

Figure 3: SDS Western Blots

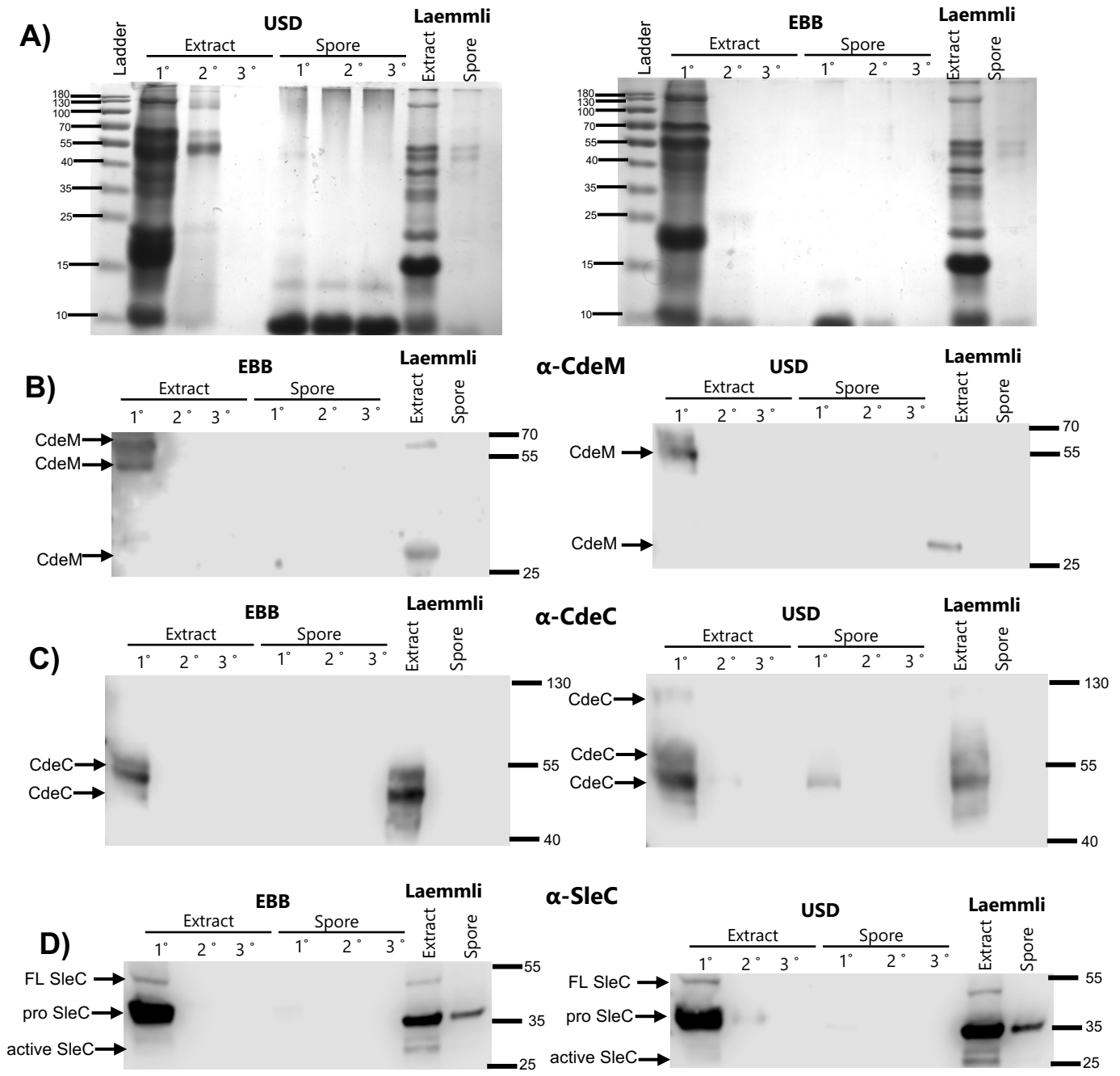

Figure 3: Phase Contrast

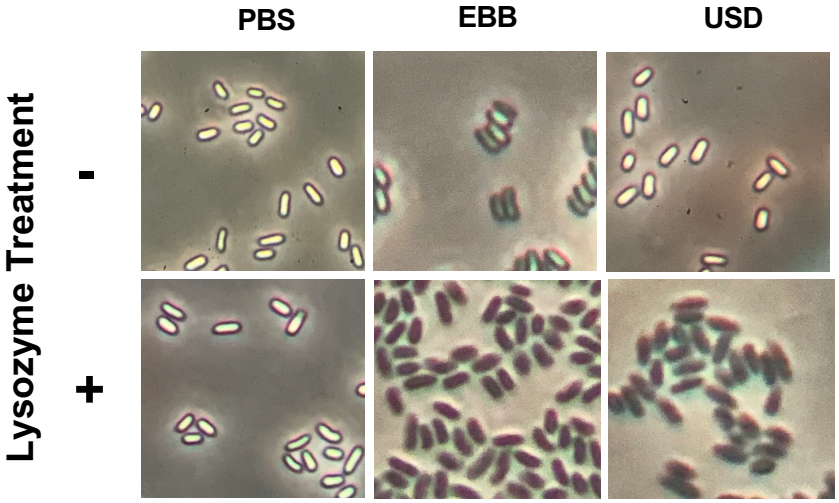
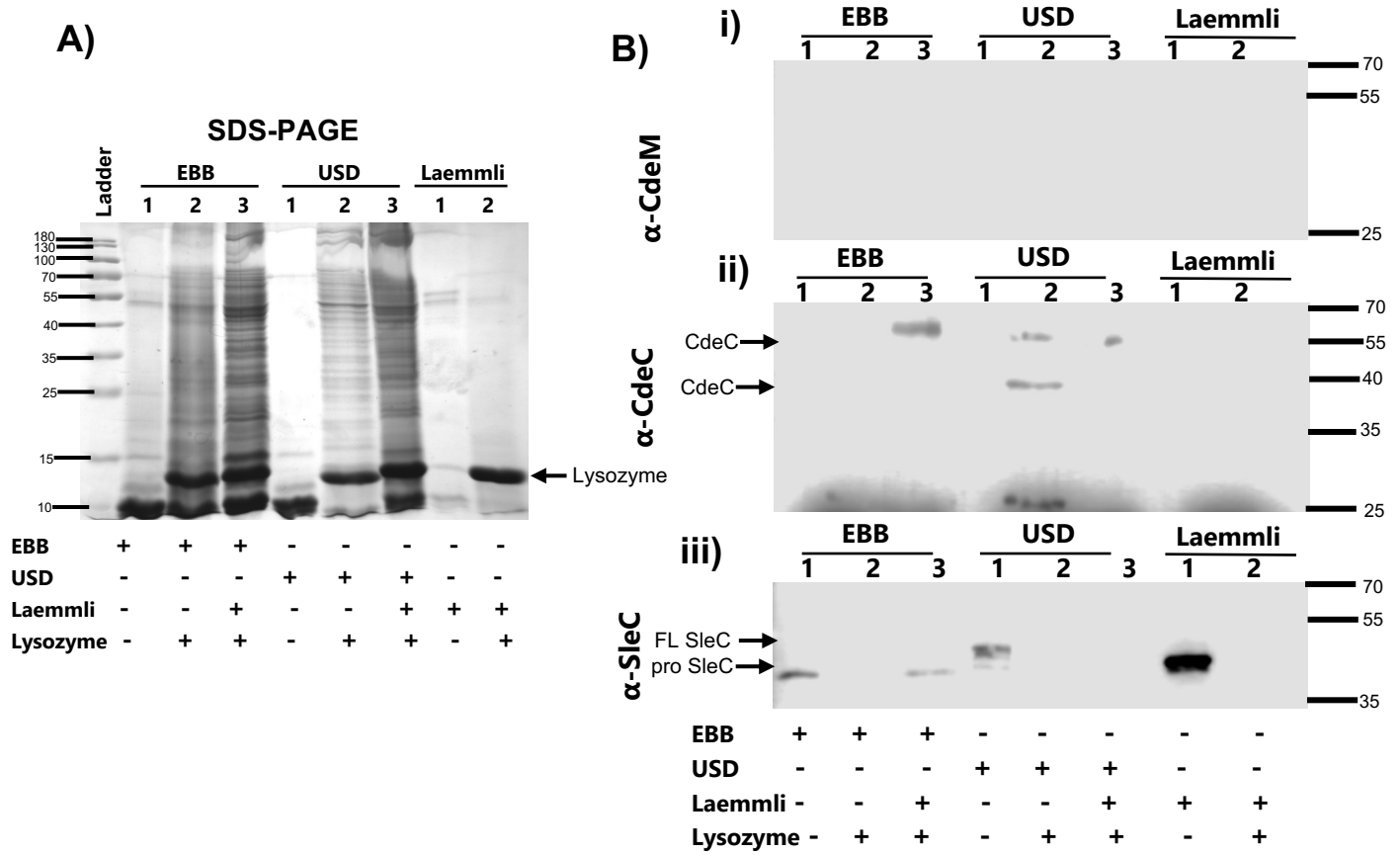
Figure 4: SDS Western Blots