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Abstract

Microbial surface contamination of equipment or of food contact material is a recurring problem in the food industry. Spore-forming bacteria are far more resistant to a wide variety of treatments than their vegetative forms. Understanding the mechanisms underlying decontamination processes is needed to improve surface decontamination strategies against endospores potentially at the source of foodborne diseases or food-spoilage. Pulsed light (PL) with xenon lamps delivers high-energy short-time pulses of light with wavelengths in the range 200 nm-1100 nm and a high UV-C fraction. *Bacillus subtilis* spores were exposed to either PL or to continuous UV-C. Gel electrophoresis and western blotting revealed elimination of various proteins of the spore coat, an essential outer structure that protects spores from a wide variety of environmental conditions and inactivation treatments. Proteomic analysis confirmed the elimination of some spore coat proteins after PL treatment. Transmission electron microscopy of PL treated spores revealed a gap between the lamellar inner spore coat and the outer spore coat. Overall, spores of mutant strains with defects in genes coding for spore coat proteins were more sensitive to PL than to continuous UV-C. This study demonstrates that radiations delivered by PL contribute to specific damage to the spore coat, and overall to spore inactivation.

Keywords

Decontamination, UV, proteins, proteomics, microscopy

1. Introduction

The pulsed light (PL) technology inactivates harmful or food spoiling microorganisms contaminating surfaces and clear liquids. PL delivered by commonly used xenon lamps consists of short (typically < 250 ms) and intense flashes of white light (200 nm–1100 nm wavelengths) rich in ultraviolet (200 nm–400 nm wavelengths) (Garvey and Rowan, 2019; Gomez-Lopez and Bolton, 2016; Gomez-Lopez et al., 2007). PL delivers short-time light pulses that are several thousand times more intense than UV-C delivered by a continuous source. The ability of PL to kill microorganisms is well documented (Kramer et al., 2017). Nevertheless, understanding intrinsic PL mechanisms leading to inactivation of resistant forms of bacteria, such as endospores, could improve PL technology and expand applications.

Spores of bacteria are markedly more resistant than vegetative cells to adverse conditions that prevail in natural or industrial environments (Setlow, 2007). This resistance favours spore dispersion and transfer to food processing facilities. Elimination of bacterial spores in industrial environments contributes to prevention of foodborne poisonings or food spoilage. Technologies have been developed over years either to inactivate bacterial spores or to decontaminate surfaces in contact with foods, such as processing equipment or packaging material (Carlin, 2011; Setlow, 2014; Soni et al., 2016; Wells-Bennik et al., 2016). Chemical biocides, such as chlorine derivatives, peracetic acid or hydrogen peroxide, are highly efficient against bacterial spores (Gopal et al., 2015; Maillard, 2011). Nevertheless, residues left after biocide application are suspected of detrimental effects on environment or health (Carrasco and Urrestarazu, 2010; Russell, 2003). UV-technologies are an alternative to chemical biocides for surface treatment

58 and inactivation of spore-forming bacteria. Continuous UV-C technology (low-pressure mercury
59 lamps emitting at 254 nm) requires relatively long exposure (typically few minutes) for spore
60 inactivation. Pulsed light (PL) is capable of achieving similar spore inactivation as continuous UV-
61 C (Gomez-Lopez et al., 2007; Koutchma et al., 2009; Levy et al., 2012) with much shorter
62 exposure times (Garvey et al., 2014) and therefore of accelerating high-flow industrial chains. PL
63 achieves up to 6 log-reduction on spores of many bacterial species with only one or two light
64 pulses (Levy et al., 2012). Application of PL on plastic polymers does not seem to generate
65 chemical residues or migrants (Castillo et al., 2013).

66 Resistance of spores lies in their specific structural organization. Spores are made of the
67 concentric layers of the coat and cortex surrounding the spore core. Spore core is characterized
68 by low water content, high content in dipicolinic acid and chelated divalent metal ions, and
69 small acid soluble proteins (SASPs) bond to DNA. DNA repair during outgrowth along with
70 SASPs, low water content of spore core and pigments allow spores to resist to UV treatments
71 (Nicholson et al., 2000; Setlow, 2014). In *Bacillus subtilis* the spore coat mainly consists of an
72 assembly of at least 70 proteins organized in a multi-layered structure (McKenney et al., 2013).
73 From the inside out, spore coat layers are the inner spore coat, the outer spore coat and the
74 crust (Imamura et al., 2011; McKenney et al., 2013). Several morphogenetic proteins, including
75 SpoIVA, SpoVID, SafA, CotE and CotXYZ, participate in the assembly of coat layers (McKenney
76 and Eichenberger, 2012; Ozin et al., 2000; Roels et al., 1992; Zheng et al., 1988).

77 Spore coat has a major role in protection against oxidative damage caused by oxidizing agents
78 (Moeller et al., 2011; Riesenman and Nicholson, 2000), or also caused by UV-A (Pattison and
79 Davies, 2006), and possibly, as shown in our previous work, against damage caused by PL

(Esbelin et al., 2016). For instance, spores of a CotE defective mutant (lacking some spore coat external layers) or chemically decoated spores were more sensitive to PL than wild-type (WT) or than untreated spores, while difference was marginal after exposure to a continuous UV-C source. More generally, spore coat proteins are not identified as major factors of resistance to UV-C (Nicholson et al., 2000; Setlow, 2014). This difference in sensitivity to PL and to monochromatic UV-C source (emitting mostly at 254 nm) could be due to a wider range of UV wavelengths emitted by PL and to higher irradiance (more than 1000 W/cm^2 with PL compared to 1×10^{-3} W/cm^2 with low-pressure mercury lamps). Our work is aimed at understanding the origin of damage caused by different UV-technologies on bacterial spores. Specifically our objectives were to determine (i) whether PL has a specific degradation effect on coat proteins of *Bacillus subtilis* using both biochemical and proteomic approaches and (ii) whether there is a specific role of spore coat proteins in resistance to PL using several *B. subtilis* strains with mutations in genes coding for coat proteins.

2. Materials and Methods

2.1. *Bacillus subtilis* strains used and preparation of spores

Origin and properties of *B. subtilis* strains 1A1 and PY79, wild-type background initially *B. subtilis* 168, and derived mutants strains are detailed in Table 1. Strains were routinely cultivated on Luria-Bertani (LB) (Biokar, Beauvais, France) agar supplemented with antibiotic concentrations recommended by donator (See Table 1) to ensure strain purity. Then, one colony was picked, grown overnight at 30°C in LB and 200 µl were plated on double-strength Schaeffer sporulation

101 (2xSG) agar without antibiotics (Nicholson and Setlow, 1990). Plates were incubated for 7 days
102 at 30°C before spore harvest. Spore suspensions were independently prepared (at least three
103 replicates of each strain) and resistance of each suspension to PL and UV-C was tested. Spores
104 were purified by repeated washing as previously described (Levy et al., 2011). Spore
105 preparations were checked to be free (< 5 %) of growing cells, germinated spores and cell debris
106 using phase contrast microscopy under 1000x magnification (Olympus BX50, Rungis, France).
107 Purified spores were suspended in demineralized sterile water, heat-treated at 70°C for 10 min
108 to inactivate vegetative cells and stored in the dark at 4°C until use. Spore counts were
109 determined by spreading 100 µl volumes of appropriate decimal serial dilutions on duplicate LB
110 agar plates incubated at 30°C for 48 h. All spore suspensions contained 10^8 to 10^9 spore cfu/ml
111 and were checked at regular time intervals for retaining phase-brightness.

112 Table 1. *Bacillus subtilis* strains used in the present work

B. subtilis strain (*designation*)	Genoype	Properties	Origin (donor)	Reference
1A1 (*wild-type*, WT)	168, *trpC2*	Wild-type strain	BSCG[a]	
1A184 (*cotA*)	*cotA1 trpC2*	Deletion in *cotA* coding for a spore coat protein. Brownish pigmentation	BSCG	(Hullo et al., 2001)
1L45 (*gerE*)	*(φ105J45) - (gerE36)+ trpC2*	Incomplete spore coat structure	BSCG	(Moir, 1981)
1S105 (*cotE*)	*cotEΔ::cat trpC2*	Spores lacking the outer spore coat layer	BSCG	(Zheng et al., 1988)
1S46 (*spoIVA*)	*pheA12 spoIVA178*	SpoIVA is a global spore coat morphogenic protein	BGSC	(Coote, 1972)
AH1910 (*spoVID*)	*trpC2 metC3 spoVID : : pJMXE*	SpoVID is the basement morphogenic protein of the spore coat structure	A.O. Henriques	(Costa et al., 2006)
AOB68 (*safA*)	*trpC2 metC3 safA : : pOZ83*	SafA is a morphogenic protein involved in assembly of inner spore coat layer	A.O. Henriques	(Ozin et al., 2000)
MTB902 (*cotB*)	*trpC2 cotB : : pMutin3*	CotB is an outer spore coat component	H. Takamatsu	
1S103 (*cotC*)	*cotC::cat trpC2*	CotC is an outer spore coat component	BGSC	(Donovan et al., 1987)
1S104 (*cotD*)	*trpC2 cotD::cat*	CotD is a spore spore coat component	BGSC	(Donovan et al., 1987)
1S107 (*cotF*)	*trpC2 cotF::cat*	CotF is an inner spore coat component	BGSC	(Cutting et al., 1991)

B. subtilis strain (*designation*)	Genoype	Properties	Origin (donor)	Reference
1A1 (*wild-type*, WT)	168, *trpC2*	Wild-type strain	BSCG[a]	
MTB907 (*cotG*)	*trpC2 cotG : : pMutin3*	CotG is a spore coat protein that participates to the assembly of CotH (and CotB) protein in the outer spore coat layer	H. Takamatsu	(Kuwana et al., 2004)
MTB908 (*cotH*)	*trpC2 cotH : : pMutin3*	CotH is an outer spore coat component	H. Takamatsu	(Kuwana et al., 2004)
1S88 (*spoVIA*)	*spoVIA513 trpC2*	SpoVIA is an outer spore coat component	BGSC	(Jenkinson, 1981)
PE594 (PY79)	Strain PY79	Wild Type strain. Prototrophic derivative of *B. subtilis* 168	P. Eichenberger	
PE618 PY79- *ΔcotE*	*trpC2 cotE : : cat*	CotE is a morphogenic protein involved in assembly of the outer spore coat layer	P. Eichenberger	(Arrieta-Ortiz et al., 2015; Zheng et al., 1988)
PE620 *ΔcotXcotYZ*	*trpC2 Δ(cotXcotYZ) : : Neo*	CotX, Y and Z proteins compose the outermost spore coat layer, the crust	P. Eichenberger	(McKenney and Eichenberger, 2012)

[a]*Bacillus* Genetic Stock Center (BGSC) (Colombus, OH)

115

116 **2.2. Evaluation of spore resistance**

117 Methods used for evaluation of spore resistance were similar to the ones used in previous work
118 (Esbelin et al., 2016; Levy et al., 2012). Briefly spores were diluted to a A_{600} of 1.0 (10^8
119 spores/ml), heat-treated for 10 min at 70°C, then cooled and kept on ice to prevent germination
120 during experiments. For UV and PL resistance, volumes of 100 µl of 6-7 decimal serial dilutions
121 of spore suspensions were spread with a rake on LB agar plates of 9 cm diameter. Treatments
122 immediately followed inoculum spreading. The number of spores on plates was therefore
123 comprised between approx. 10^7 and 1 spore cfu/plate. Phase-contrast microscopy at 1000x
124 magnification did not reveal any spore that turned phase-dark and therefore having germinated
125 within 2.5 min, i.e. longer than time between spreading and exposure. The distribution of
126 spores on the agar surface was homogeneous and spore shielding was not detected. PL and
127 continuous UV-C exposure of inoculated agar plates were performed within 30 s - 2 min
128 following spreading. LB agar plates were placed under radiation sources. Inoculated plates were
129 incubated at 30°C for at least 48 h prior to colony counting to ensure that all survivors had
130 formed visible colonies. Untreated samples were used as controls.

131 **2.3. Pulsed light and continuous UV-C devices**

132 PL resistance of *B. subtilis* spores was tested with a previously described lab-scale equipment
133 (Claranor, Avignon, France) (Levy, 2010; Levy et al., 2012). This device delivers pulses (duration
134 250 µs) of polychromatic light (wavelengths between 200 nm and 1100 nm) produced by a
135 xenon flashlamp containing 18.5 % of fluence corresponding to UV (wavelengths between 200

nm and 400 nm) under a charging voltage of 2500 V. The emission spectrum of the xenon lamps is shown in supplementary Fig 1. The UV-C proportion in PL was determined with spectrophotometric methods (Claranor, unpublished data). PL fluence varied from 0.3 J/cm² to 10.8 J/cm² (from 0.3 J/cm² to 1.8 J/cm² for survival curves) according to the number of delivered flashes (one to 16 at 1 s interval) and the distance to the lamp (between 8 cm and 25 cm). Unflashed samples were used as controls. UV-C resistance was measured by using a UV-C chamber equipped with three low-pressure mercury lamps (2.5 cm-diameter and 43.5 cm-long) (model TUV-T8, 15 W, Philips, The Netherlands) placed in parallel at approx. 45 cm from the target and emitting short-wave radiations with a peak at 253.7 nm according to manufacturer's specifications. The irradiation strength of 1.07 mW/cm^2 was measured with a radiometer (model VLX-3W, Vilber Lourmat, Marne-la-Vallée, France). Tested UV-C fluence ranged from 0.027 to 0.88 J/cm^2 (from 0.027 J/cm² to 0.15 J/cm² for survival curves) and were obtained by times of treatment ranging from 25 s to 13 min 48 s (to 2 min 30 s for survival curves). Viability reduction was expressed as $\log_{10} N_0/N$ as a function of the applied fluence, where N_0 is the initial count of the spread suspensions in cfu/plate and N the number of survivors in cfu/plate.

151 **2.4. SDS PAGE of coat protein extracts**

152 Samples (100 µl) of purified spores (at A_{600} = 3.0) of wild-type strain 1A1 were mixed with 14 ml
153 of sterile demineralized distilled water and poured into Petri dishes of 9 cm diameter giving a 3
154 mm deep water layer. Spores were then exposed to either PL (fluence between 1.8 J/cm^2 to
155 10.8 J/cm^2) or UV-C (fluence between 0.15 J/cm^2 and 0.88 J/cm^2). Exposed spores were
156 transferred to a new test tube and harvested by centrifugation (7000 X *g* for 5 min at 4°C).
157 Spore pellets were suspended again in 40 µl of SDS PAGE loading buffer (Biorad, Munich,
158 Germany) and boiled for 5 min, mixed vigorously for 1 min and boiled again for 5 min. Samples
159 were briefly centrifuged and 15 µl (approximately 40 µg of total proteins) were fractionated
160 using a large format 1-D electrophoresis systems (protean II Biorad) on a 12 % acrylamide gel
161 containing SDS (SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis). Gels
162 were stained with Coomassie brilliant blue R-250, and then destained.

163 **2.5. Western blot**

164 Samples were resolved by SDS-PAGE and electro-transferred onto nitrocellulose membranes
165 using the iBlot Dry Blotting System according to standard procedures (Invitrogen, Illkirch,
166 France). Membranes were probed with the appropriate antibody. Blotted membranes were
167 developed with a 1:15000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G
168 (Sigma, Saint-Quentin Fallavier, France) and an enhanced chemiluminescence substrate
169 (SuperSignal West Femto Chemiluminescent Substrate).

170 **2.6. Proteomic analysis**

For proteomics, a 100 µl sample of each spore suspension was treated as follows. The spores were rinsed by suspension in 5 mL of cold sterile demineralized water and agitation with an orbital shaker for 5 min at 4°C to remove loosely attached proteins. The spore pellet was obtained by centrifugation of the suspension at 3500 x *g* and the supernatant was discarded. This procedure was twice repeated. The spores were then suspended in 1X NuPAGE LDS sample buffer (Invitrogen) supplemented with DTT (at 100 mmol/l final concentration) (Sigma) for protein solubilisation and loaded onto the different wells of one 4–12 % gradient NuPAGE gel (Invitrogen). The gel was operated with MES buffer, run at 150 V (Invitrogen) for 5 min resulting into a short gel migration (5-8 mm). For each sample, the whole protein content from each well was excised. Their protein contents were then treated with DTT and iodoacetamide and then proteolysed with trypsin and proteasMAX^{TM} (Promega, Madison, WI, USA) and analysed by LC-MS/MS as previously described (Clair et al., 2012). Briefly, digested peptides were loaded and desalted on line on a reverse-phase precolumn (C18 PepMap 100 column, LC Packings). Peptides were separated using a 90 min gradient from 5 % to 60 % solvent B (0.1 % HCOOH / 80 % CH_3CN) in solvent A (0.1 % HCOOH / 100 % H_2O), at a flow rate of 0.3 µl/min and analysed with an LTQ-Orbitrap XL mass spectrometer (ThermoFisher, Illkirch, France) in data-dependent mode using the TOP7 strategy.

2.7. Proteomic data analysis

The generated raw files were analysed in MaxQuant (Cox et al., 2014) as previously described (Clair et al., 2016). Briefly, the false discovery rate was set at 0.01 at the spectral, peptide and protein levels. Proteins were identified with at least two tryptic or semi-tryptic peptides of a minimum length of six amino acids by searching against the UniProt *Bacillus subtilis* strain 168

193 database (UniProtKB, downloaded in 2018). Carbamidomethylation was set as fixed
194 modification and N-terminal acetylation and oxidation of methionine. The match between run
195 algorithm was used and the unmodified intensities (note: LFQ intensities were not used, as they
196 comprise some global normalization steps that would have impaired the cumulative intensity
197 values). Normalization and statistical test were performed with the 'stat' package of R. The
198 intensities were then log_2 transformed and median centred normalized within a given sample
199 group (e.g. the samples treated with 1.8 J/cm^2 were median centred between them but not
200 against untreated control samples). Only proteins with measured intensities in at least 2-3
201 replicates of a given condition were considered for quantification and missing values were
202 imputed using the minimal normalized intensity of the table divided by two. Two-tailed
203 distribution heteroscedastic Student's t-tests were used to identify proteins with lower
204 abundance in treated samples.

205

206 **2.8. Transmission Electron Microscopy**

207 Freshly prepared spores were centrifuged at 3500 X *g* during 5 min and fixed for 2 h at room
208 temperature with 2.5 % glutaraldehyde (v/v) in a 0.1 mol/l sodium cacodylate buffer (pH 7.1)
209 containing 0.5 mg/ml ruthenium red. Spores were washed by three centrifugations (5 min at
210 3500 X *g*) in 0.2 mol/l sodium cacodylate and fixed for 1 h at room temperature with 2 %
211 osmium tetroxide. Next, samples were washed three times with water. After centrifugation,
212 pellets were embedded in 3 % (wt/v) agar and submitted to successive agitated dehydration
213 baths with increasing concentrations of ethanol (30 %, 50 %, 70 %, 90 %, and 100 %). Ethanol

214 was replaced with propylene oxide and sequentially exchanged with araldite resin. Samples
215 were polymerized for 48 h at 60°C. Thin sections (60 - 80 nm thick) were obtained with an
216 ultramicrotome (Leica) and stained with 32 % uranyl acetate and lead citrate and were observed
217 by transmission electron microscopy (TEM) (TE Microscope FEI-Philips CM10). Thin sections of
218 at least 100 spores of one batch of spores were carefully examined with TEM by two
219 experimenters to detect possible differences in structures between control unexposed and PL-
220 exposed spores of *B. subtilis* strain 1A1 (fluence 7.1 J/cm^2 delivered in 8 flashes). Representative
221 micrographs of the observed differences were selected.

222

223 **2.9. Statistical analysis**

224 Parameters describing inactivation of spores as a function of PL or UV-C fluence were calculated
225 for each survival curve. Fluence leading to 3-fold log reduction (F_3) (Albert and Mafart, 2005)
226 was estimated using non-linear mathematical functions and Microsoft® Excel® 2010 solver
227 function. R_{max} was the observed $\log_{10}$-reduction at the highest tested fluence. TMean values and
228 standard error (SE) of the mean were calculated from data obtained from at least three
229 independent experiments (different dates, different cells cultures and spore preparations). The
230 significance of differences between strains 1A1 or PY79 and derivative mutants was determined
231 by a Student's t-test using the Microsoft® Excel data Analysis tool.

232

3. Results

3.1. Elimination of *B. subtilis* spore coat proteins by PL

Spores of *B. subtilis* strain 1A1 were exposed to increasing fluence emitted by PL or a continuous UV-C source (λ = 254 nm). Spores were rinsed in cold water to remove loosely attached proteins. Coat proteins were extracted, then separated by SDS-PAGE (see section 2.4). A fluence of 1.8 J/cm^2 delivered by PL caused a 2-3 log reduction in spore counts; PF fluence of 7.1 J/cm^2 or 10.8 J/cm^2 caused a log-reduction greater than 6. A UV-C fluence of 0.15 J/cm^2 delivered by the continuous UV-C source caused a 4-log reduction; UV-C fluence of 0.59 J/cm^2 or 0.88 J/cm^2 caused a log-reduction greater than 6. Controls were spore coat proteins extracts from unexposed spores. Number and intensity of electrophoresis gel bands of proteins of molecular weight (MW) between 10 kDa and 50 kDa remained unchanged whatever UV-C fluence. In contrast, intensity of bands of almost all 10 kDa - 50 kDa MW proteins decreased as PL fluence increased (Supplementary Fig 2).

Proteins of spores treated by PL at a fluence of 1.8 J/cm^2 or by UV-C at a fluence of 0.15 J/cm^2 were analysed by western blotting using antibodies targeted against spore coat proteins CotA, CotSA, CotC, CotE, CotH, and CotJ of MW comprised between 25 kDa and 70 kDa (Fig 1 and supplementary Fig 3). The intensity of bands of untreated and UV-C treated spores was highly similar for all tested coat proteins. In contrast, binding to their specific antibodies of spore coat proteins from PL-exposed spores showed diverse patterns. Strikingly, while the applied PL fluence resulted in a lower reduction in spore counts than the applied UV-C fluence, there was a

254 total disappearance on western blots of immunoreactive bands of anti-CotA and anti-CotSA
255 antibodies against the PL-exposed spore protein extract. Binding of anti-CotC and anti-CotE
256 antibodies was slightly lower for the PL-exposed spore coat protein extract than for the ones of
257 the control. Binding with anti-CotJC and anti-CotH antibodies on PL-exposed and control spore
258 protein extract was highly similar. Nevertheless, cotH abundance was lower in PL-treated spores
259 (Fig. 2). Overall, these differences in patterns of immunoreactive bands of coat proteins from
260 spores exposed to PL or UV-C suggests that (i) PL has a stronger effect than UV-C on coat
261 proteins located within the spore, and (ii) that effects of PL may be markedly different for
262 diverse epitopes/proteins (Fig 1). The disappearance of bands on electrophoresis gels or of
263 some bands on western blots could be either due to (i) a detachment of coat proteins from
264 spore as direct consequence of treatment and elimination by washing steps or (ii) of
265 degradation of proteins, at least of site binding antibody.

266

267 **3.2. Proteomic analysis of proteins extracted from *B. subtilis* spores treated by PL**

268 To evaluate in depth how PL treatment affects coat proteins, proteome of coat of *B. subtilis*
269 spores exposed to PL at fluence of 1.8 J/cm^2 or 7.1 J/cm^2 (the latter resulting in a log reduction
270 greater than 6) was analysed and compared to coat proteome of non-exposed spores. Same
271 amount of either treated or untreated (control) spores was rinsed in cold water to remove
272 potentially detached proteins. Then coat proteins were extracted and loaded on a SDS page gel.
273 After a short migration of proteins in the gel, the whole protein content from each well was

sampled as a sole band and digested with trypsin. Resulting peptides were analysed by LC-MS/MS (See section 2.6).

When only 2.9 % of genes are known to code for spore coat proteins (Abhyankar et al., 2015; Abhyankar et al., 2016; Lai et al., 2003; McKenney et al., 2010; McKenney and Eichenberger, 2012), over 79 % of the 34,936 spectra attributed to *B. subtilis* proteins (i.e. 27,644 spectra) were belonging to proteins previously identified as located in spore coat, therefore demonstrating that the preparation protocol efficiently enriched for coat proteins (Supplemental datasets 1-2). These spectra were attributed to 1,157 *B. subtilis* peptides (Supplemental dataset 3). In order to determine whether the global amount of detected peptides was affected by PL treatment, peptide intensities attributed to *B. subtilis* proteins were summed within each sample and compared. As shown in Fig 2A, summed peptide intensities were more than 2-fold lower in treated samples than in untreated control (Student's t-test *p*-value < 0.05). Furthermore, the proportion of the signal attributed to peptides not resulting from trypsin digestion (i.e. non-tryptic peptides) was significantly increased (Student's t-test *p*-value < 0.01) in treated samples (Fig 2B). Altogether, these results suggest that PL reduces the amount of spore coat proteins likely by cleaving amino acid bonds. In total 149 *Bacillus subtilis* proteins were identified (i.e. with at least two peptides and a false discovery rate < 1 %). Proteins intensities were $\log_2$ transformed and normalized within each treatment group (to avoid an artificial increase for treated samples of protein amount). The proteomics profile data were classified by applying a principal component analysis (PCA) (Fig 2C). First principal component (PC1) explained 49 % of the overall variance and showed a clear discrimination of PL-treated and untreated samples. Neither second component nor third component of PCA

296 explaining 18.0 % and 12.5 % of the variance respectively (data not shown) distinguished the
297 two treatments. The abundance of 59 proteins was significantly lower in treated samples than
298 in controls (Student's t-test, *p*-value < 0.05) (Fig 2D and Supplemental dataset 4). These 59
299 proteins include 38 proteins known to be part of spore coat. In most instances, the two applied
300 PL fluences reduced abundance of the same proteins. As expected from western blots, CotA,
301 CotSA, CotC and CotE were reduced in abundance in treated samples and abundance of CotJC
302 was not significantly affected by PL (Supplementary Fig 4). Conversely, while western blot
303 analysis suggested that abundance of CotH in spores was not affected by PL, proteomics
304 measurement indicates the opposite, as indicated in section 3.1. This discrepancy either could
305 result from a lack of specificity of the CotH antibody, or from a degradation, revealed by
306 proteomic analysis of CotH, that does not affect the epitope targeted by the anti-CotH antibody.
307 Interestingly, among spore coat proteins reduced in abundance were some proteins belonging
308 to diverse layers of coat including crust component CotY, outer coat proteins CotE, CotA and
309 CotS (McKenney et al., 2013), or inner coat proteins SafA (Ozin et al., 2000) and YaaH (Imamura
310 et al., 2010). Overall proteome analysis suggests a global degradation or detachment after PL
311 treatment of proteins composing spore coat.

312

313 **3.3. Inactivation of spores of *B. subtilis strains* 1A1 or PY79 and of isogenic mutants with**
314 **defects in genes coding for spore coats**

315 Fig 3 shows typical survival curves of WT strain and derivative mutants exposed to PL. Survival
316 curves were sigmoidal in most instances. The $\log_{10}$ reduction at the highest tested UV-C fluence

317 was comprised, for spores of all strains, between 2.6 and 5.7. The $\log_{10}$ reduction was 1.9 at the
318 highest tested PL fluence with spores of *B. subtilis* strain 1A1 and between 1.4 and 5.3 for
319 spores of its isogenic strains (data not shown). The $\log_{10}$ reduction was 4.8 at the highest tested
320 PL fluence with spores of *B. subtilis* strain PY79 and between 3.2 and 6.0 for spores of its
321 isogenic strains (Fig 4). Curves fitted with the model of Albert and Mafart (Albert and Mafart,
322 2005) satisfactorily followed experimental data for both PL and continuous UV-C treatments and
323 spores of all tested strains, as illustrated for instance in Fig 3. Fluence leading to a three-fold log
324 reduction (F_3) and maximal $\log_{10}$ reduction for the highest tested fluence (R_{max}) were estimated
325 for each survival curve (Fig 4). Typical inactivation patterns were observed. Spores of some
326 strains, such as *cotG*, behaved similarly to WT spores, with no significant difference for both F_3
327 and R_{max} ($p > 0.25$). In contrast, spores of strains *spoVID* and *cotE* showed a marked difference in
328 inactivation (Fig 3), illustrated by R_{max} and F_3 values significantly different compared to the ones
329 of the WT ($p < 0.05$) (Fig 4). Both parameters R_{max} and F_3 were descriptors of sensitivity of spores
330 of the two WT strains and of the 15 derivative-mutant strains with defects in spore coat-
331 proteins (Fig 4).

332 Overall, there were more derivative-mutant strains with spores showing a higher sensitivity to
333 PL than to continuous UV-C. Spores of 10 of these strains exposed to PL showed a significantly
334 higher sensitivity than WT strains, according to their R_{max} values while spores of only three
335 strains exposed to continuous UV-C were more sensitive. In the same way, F_3 of spores of six
336 strains exposed to PL were significantly lower than F_3 of WT ($p < 0.05$), while F_3 of spores of only
337 one strain exposed to continuous UV-C was significantly lower than F_3 of WT. Among those
338 strains, F_3 values and R_{max} values of spores of strains *spoIVA*, *spoVID*, *safA*, *cotE*, *cotF*, and

339 *spoVIA* were significantly different of the ones of WT. Spores of none of the strains exposed to
340 UV-C were significantly affected for both R_{max} and F_3 when compared to the WT strains. Spores
341 of some strains, *cotB*, *cotD*, *cotG* and *cotH,* were not affected in their sensitivity to PL.
342 Surprisingly R_{max} of spores of PY79 *cotXYZ* was lower than R_{max} of spores of WT PY79 strain when
343 exposed to both PL and continuous UV-C, suggesting a higher resistance to both treatments.
344 Spores of a few strains showed higher R_{max} and/or F_3 values than the ones of WT when exposed
345 to continuous UV-C.

346

347 **3.4. Changes in coat arrangement in PL-exposed spores**

348 Thin sectioning transmission electron micrographs (Fig 5) reveal differences in ultrastructure of
349 control spores and spores exposed to PL at a fluence of 7.1 J/cm^2 resulting in log-reduction
350 greater than 6. A gap between lamellar inner coat and outer coat and its electron dense
351 striations appears on at least 80 % of observed PL-exposed spores.

352

353

4. Discussion

Previous works have clearly established that UV-C wavelengths play a major role in the killing effect of PL on micro-organisms. Filtering-out UV-C radiations dramatically decreases PL efficiency (Bohrerova et al., 2008; Levy et al., 2012; Takeshita et al., 2003). Does this mean that PL causes the same damage as continuous UV-C sources or that mechanisms implicated in resistance to PL or to UV-C are identical? This question has been previously addressed and protein leakage likely consecutive to membrane damage caused by PL and not observed on UV-C treated has been reported for instance on bacteria or yeast cell (Garvey et al., 2016; Kramer et al., 2017; Takeshita et al., 2003). PL applies a polychromatic source of very high energy with wavelengths between 200 nm and 1100 nm. Irradiance of PL sources is several orders of magnitude higher than irradiance of continuous UV-C sources. In the present work, we compared damage caused by PL on *B. subtilis* spores to damage caused by UV-C. PL, but not continuous UV-C treatment, caused in electrophoresis gels disappearance of spore coat proteins bands. These observations were further confirmed by western-blots of specific spore coat proteins, suggesting that PL can induce their elimination by direct degradation or detachment. CotA and CotSA were not detected after exposure of spores to PL. Elimination of CotA and CotSA and more generally of a large set of other proteins including CotB, CotY, YaaH, CotD, SafA, and CotE was confirmed by proteomic analysis of spore coat (Fig 2). In this regard, TEM images revealed unbound junction segments at many locations between inner coat and outer coat because of PL treatment (Fig 5). Remarkably some proteins believed to be mainly localized in the core of the spore such as SASPs SspN and SspO (Cabrera-Hernandez et al., 1999; Cabrera-Hernandez and Setlow, 2000) as well as the 30S ribosomal protein S11 (RpsK) were also reduced

376 in abundance in PL-treated samples suggesting that PL might also impact internal spore core
377 proteins (Fig 2).

378 Subjected to damage, spore coat proteins are also deeply implicated in resistance to PL. Spores
379 of strains defective in spore coat proteins were more sensitive to inactivation by PL than to
380 inactivation by UV-C (Fig 4). This includes in particular spores of strains defective in proteins
381 SpoIVA, SpoVID, SafA, and CotE contributing to morphogenesis of the multi-layered
382 proteinaceous spore coat (McKenney et al., 2013). CotE importance was underlined in a
383 previous report (Esbelin et al., 2016) and confirmed here. Spore coat integrity was also a major
384 determinant of the resistance of *B. subtilis* spores to the diversity of Mars UV radiations
385 (Moeller et al., 2012), blue light (± 400 nm wavelength) (Djouiai et al., 2018) and to low-pressure
386 plasma, which generates a range of UV wavelengths (a characteristic of the treatment shared
387 with PL) together with reactive chemical species (Raguse et al., 2016), but not to UV-C at 254
388 nm wavelength (Riesenman and Nicholson, 2000). Absorption of radiation by spore coat and/or
389 neutralization of reactive chemical species generated by process are the main hypotheses to
390 explain higher sensitivity of spore coat-defective mutant strains (Djouiai et al., 2018; Raguse et
391 al., 2016). Spore coat proteins are also major determinants of the resistance of spores to
392 oxidative chemical biocides including peroxyacetic acid alone, or in combination with
393 supercritical CO_2, peroxides or iodine (Leggett et al., 2015; Li et al., 2017; Setlow et al., 2016;
394 Setlow et al., 2014).

395 Which properties of PL could generate damage not observed with a continuous UV-C source?
396 Photochemical damage to DNA is generally reported as a major cause of cell death (Kramer et
397 al., 2017). Photochemical damage was not examined in the present work, but is expected

398 because of the high proportion of UV in PL. Previous work already suggested that damage may
399 depend on UV light source: systems allowing DNA repair of damage to DNA are unequally
400 important for spores treated either by PL or by continuous UV-C (Esbelin et al., 2016).
401 Inactivation of micro-organisms has been observed at diverse wavelengths in the UV-C
402 spectrum, certainly to different extents. Spores of *B. pumilus* were 8-9 times less sensitive to a
403 medium pressure UV-source (with peak emission at several wavelengths in the UV-C range) as
404 wavelengths lower than 244 nm were filtered (Beck et al., 2015). In general terms, the relative
405 action spectra of UV radiation against *B. subtilis* spores do not match DNA absorption spectra.
406 Wavelengths below 250 nm are emitted at a lower intensity by the PL source used in this work
407 (Supplementary Fig 1.) but Chen et al. (2009) showed that these wavelengths are more active
408 against *B. subtilis* spores. External layers may not be the primary cause of difference in
409 absorption spectra of spores and DNA, as spectra of decoated spores matched pretty well
410 spectra of intact *B. subtilis* spores. PL has also shown photochemical effects on a range of
411 biological molecules. PL degrades mycotoxins made of conjugated heterocycles such as
412 aflatoxin, zearalenone, ochratoxin, or deoxynivalenol (Moreau et al., 2013). Proteins, in
413 particular side chains of tryptophan, tyrosine, phenylalanine, histidine, cysteine and cysteine,
414 are sensitive to photo-oxidation by UV-A and UV-B, also delivered by PL (Pattison and Davies,
415 2006). Wavelengths of absorption of peptide bonds are in the range 190 – 230 nm and light of
416 these wavelengths also emitted by PL have therefore the potential to degrade proteins.
417 Consequences of protein photo-oxidation are diverse and include unfolding, aggregation, or
418 modification of protein network organization. Analyses of UV and fluorescence spectra of milk
419 proteins specifically exposed to PL indicated protein aggregation and modification of polarity at

420 the proximity of tryptophan residues (Elmnasser et al., 2008). Additionally, photo-oxidized
421 proteins can cause secondary damage through the formation of oxidative products (Manzocco,
422 2015; Pattison and Davies, 2006). Does high energy significantly contribute to inactivation by
423 PL? Second law of photochemistry states that photochemical effect depends only on number of
424 photons absorbed and, in other words, that same inactivation can be achieved with high-
425 fluence – short time and low fluence - long-time exposures (Gomez-Lopez and Bolton, 2016).
426 For instance *B. subtilis* spore inactivation with sources emitting at ± 250 nm was only dependent
427 on fluence and independent of irradiance despite a 1: 10^8 difference between sources(Rice and
428 Ewell, 2001). Demonstrating specific effects of high irradiance remains an issue.

429 In terms of application, in addition to the acceleration of decontamination processes in food
430 industry lines, the diversity of wavelengths emitted by PL multiplies cellular targets and
431 subsequently cellular damage causing injury and death. Hence, antimicrobial efficiency of PL is
432 potentially higher than antimicrobial efficiency of other UV-radiation sources, and not solely on
433 spores of bacteria. For instance, conidiospores of *Aspergillus niger* were, at equivalent fluence,
434 strongly inactivated by PL and were almost non-affected by a continuous UV-C source (Levy et
435 al., 2012). The spectrum emitted by xenon lamps used for PL decontamination is determined by
436 lamps themselves and by operating conditions (charging voltage, pulse duration…) (Schaefer et
437 al., 2007). In other terms spectra emitted by PL could be adjusted to some extent towards
438 emission of the most efficient wavelengths. A better understanding of the relative contribution
439 of the diverse wavelengths to photochemical damage induced by PL on nucleic acids, proteins
440 and other cell components is necessary for wise evolution and design of future PL devices.

441

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Figure captions

Fig 1. Western blots with antibodies directed against spore coat proteins CotA, CotSA, CotC, CotE, CotH and CotJC after exposure of *B. subtilis* spores to a fluence of 1.8 J/cm^2 delivered by PL (allowing a 2-log reduction) or a UV-C fluence of 0.15 J/cm^2 (allowing a 4-log reduction). Numbers on the left-hand are molecular weights (kDa); U: untreated controls; PL: pulsed-light treated samples; UV: UV-C treated samples.

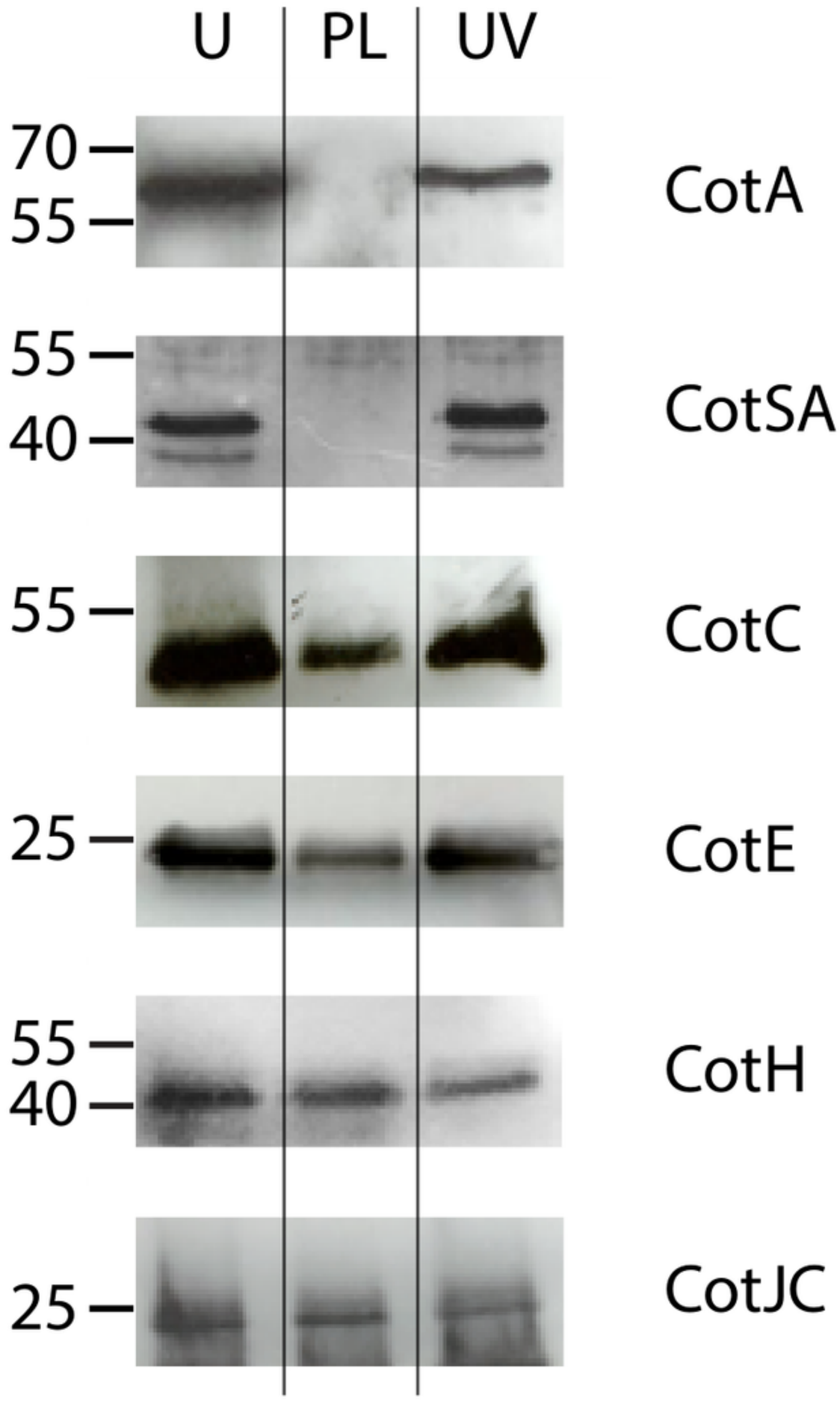
Fig 2. Proteomic characterization of *B. subtilis* strain 1A1 spores subjected to a pulsed light (PL) treatment. (A) Cumulative intensity of peptides for PL-treated and control samples. (B) Percentage of cumulative intensity attributed to semi-tryptic events. In (A) and (B) error bars represent standard error and asterisks represent Student's t-test *p*-values (***, $p < 0.001$; **, $p < 0.01$, *, $p < 0.05$). (C) Principal Component Analysis projection of untreated (orange) and PL-treated samples (blue). The first principal component separates out treated and untreated sample. Replicates are identified by numbers (1 to 3). (D) Heat map of proteins significantly changing in abundance in PL treated spore coat compared to untreated spore coat. The color scale represent $\log_2$ (reduction) of protein abundance in treated samples. Asterisks represent the *p*-value cutoffs (***, $p < 0.001$; **, $p < 0.01$, *, $p < 0.05$) of the comparison with untreated samples. The green box shows results validating western blot results (Fig 1). The black box around CotH indicates a possible decrease revealed by proteomic analysis, and conversely not observed on the western-blot (Fig 1).

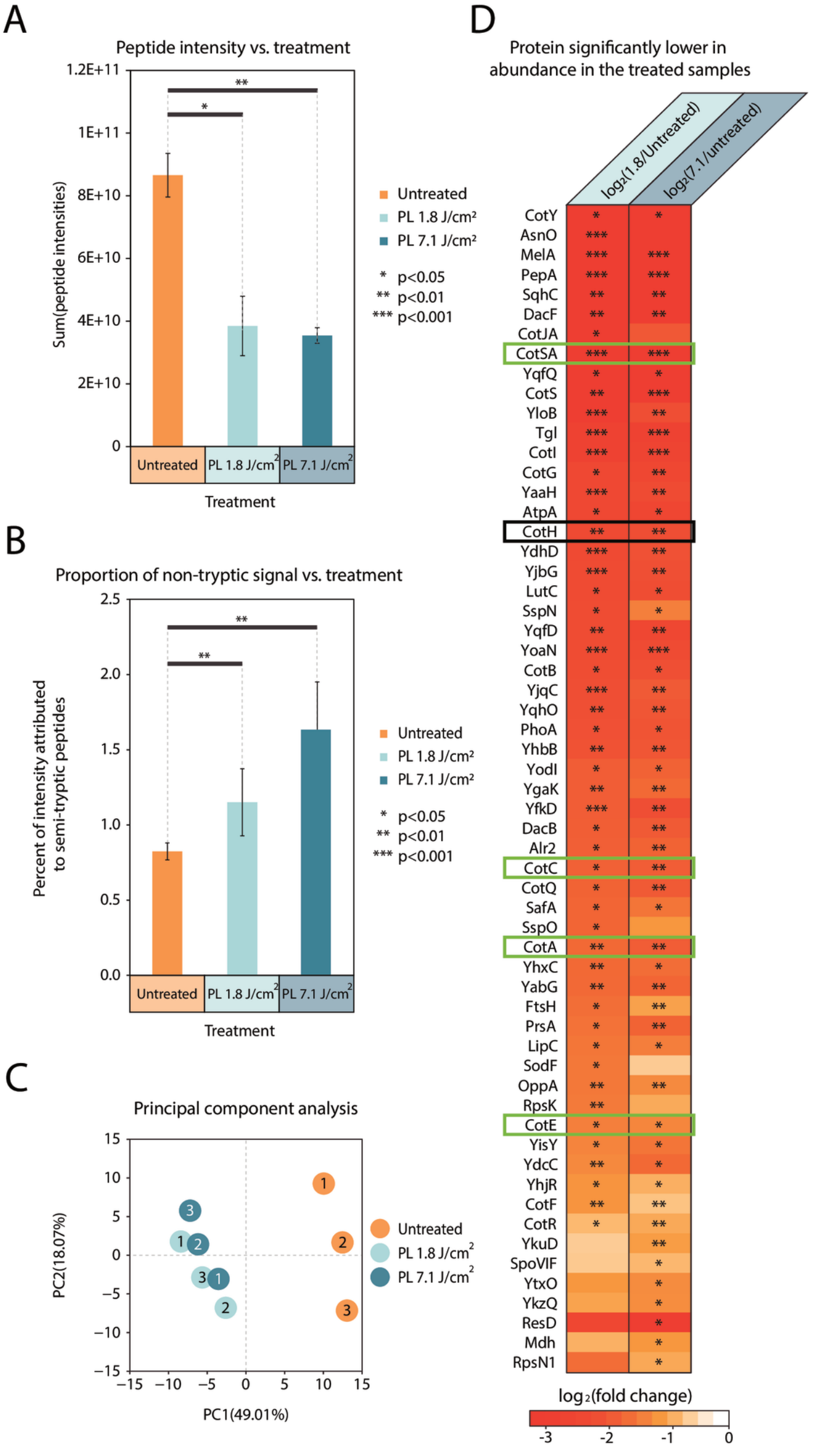
Fig 3. Inactivation by PL of *B. subtilis strain* 1A1 (◆) and of a selection of derivative mutants with defect in genes *cotG* (●), *spoVID* (▲) or *cotE* (■) coding for morphogenetic and/or spore coat proteins. Symbols show mean of at least three biological replications (different spore

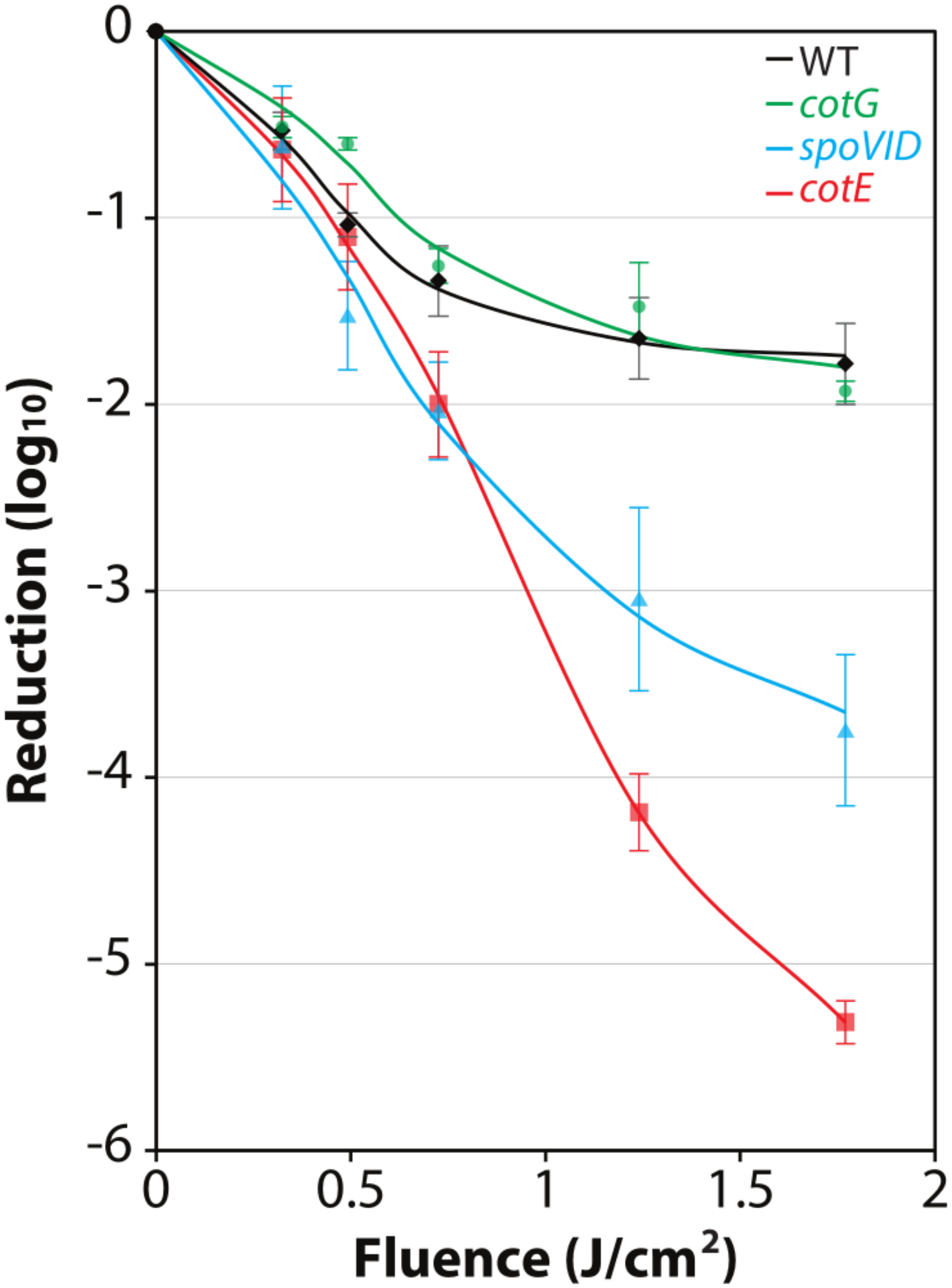
preparations). Error bars show standard error. Color lines represent curves that have the best fit to data points with the model of Albert and Mafart (2005). The model was otherwise used to determine fluence to 3 log-reduction (F_3) and log-reduction at maximal tested fluence (R_{max}) presented in Fig 4.

Fig 4. Comparison of log-reduction at maximal tested fluence (R_{max}) (A, B) and of fluence to 3 log-reduction (F_3) (C, D) between *B. subtilis* strain WT 1A1 or PY79 (in bold characters) and their respective derivative mutants with defects in genes coding for spore coat proteins. Strains were treated either with pulsed light (A, C) or with a continuous UV-C source (B, D). Histogram bars represent the arithmetic difference in R_{max} values of WT strains and respective mutants. Errors bars represent standard error ($n \geq 3$). Shaded bands surrounding vertical axes show standard error on R_{max} or F_3 values of WT strains. Negative values in R_{max} comparison graphs correspond to inactivation greater for mutant strain than for WT. Values < 1.0 in F_3 comparison graphs (C, D) correspond to inactivation greater for mutant strain than for WT. Blue histogram bars show a significant difference in R_{max} or F_3 value at $p < 0.05$. The $\log_{10}$ reduction was 1.8 at the highest tested PL fluence with *B. subtilis strain* 1A1 and between 2 and 6 for its derivative strains. The $\log_{10}$ reduction was between 4 and 5 at the highest tested PL fluence with *B. subtilis strain* PY79 and between 2 and 6 for its derivative strains.

Fig 5. Transmission electron microscopy pictures and magnification of spore coat section of *B. subtilis* strain 1A1 spores, control (A, C) and exposed to a PL fluence of 7.1 J/cm^2 (B, D). Co= spore core; Cx = spore cortex; IC = inner spore coat, OC = outer spore coat. Red arrows indicate position of gap between inner and outer spore coat observed in PL-exposed spores.

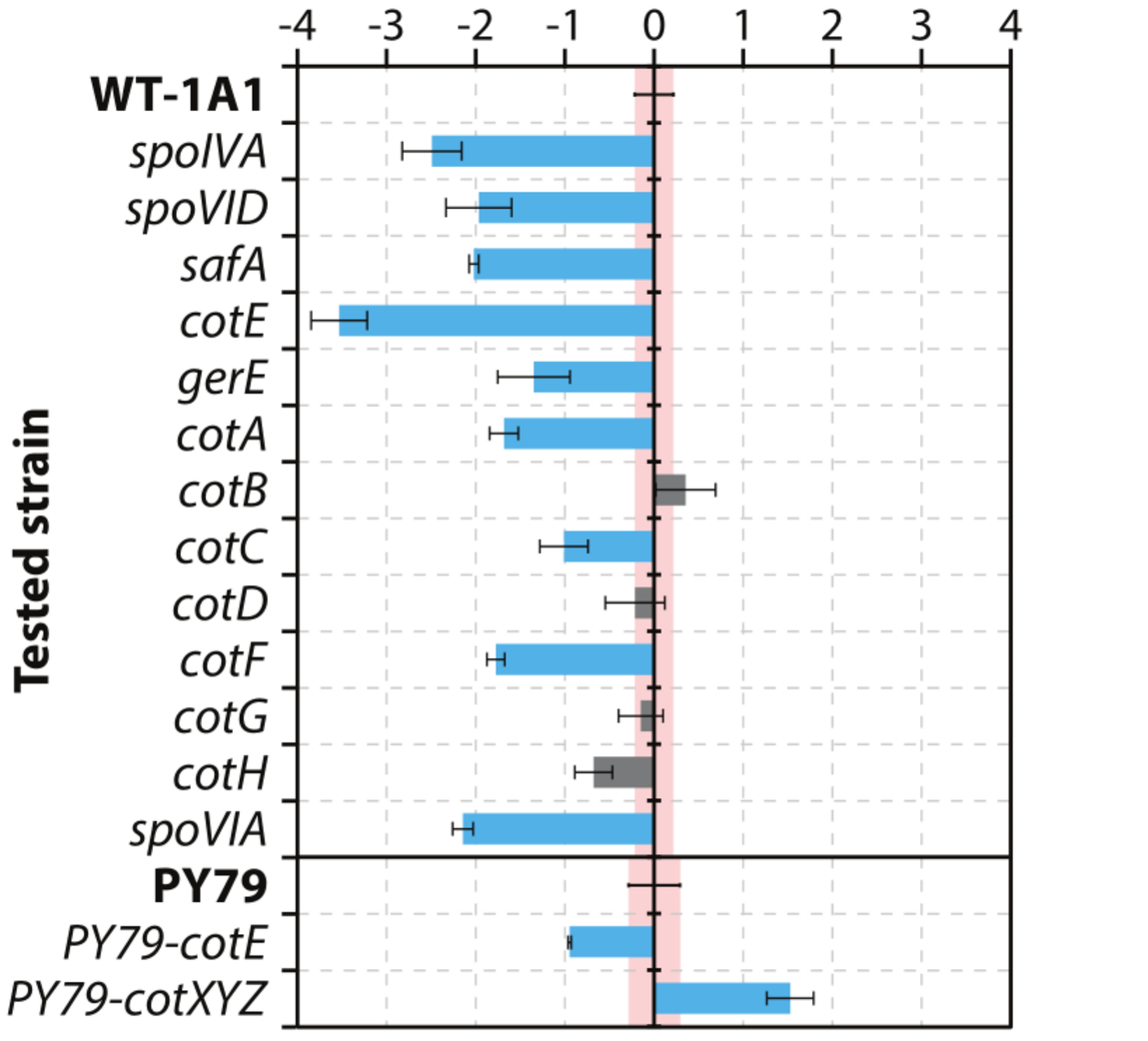

U
PL
UV
70
55
CotA
55
40
CotSA
55
CotC
25
CotE
55
40
CotH
25
CotJC


A
Peptide intensity vs. treatment
1.2E+11
1E+11
8E+10
6E+10
4E+10
2E+10
0
Sum(peptide intensities)
**
*
Untreated
PL 1.8 J/cm²
PL 7.1 J/cm²
Treatment
Untreated
PL 1.8 J/cm²
PL 7.1 J/cm²
* p<0.05
** p<0.01
*** p<0.001
B
Proportion of non-tryptic signal vs. treatment
2.5
2.0
1.5
1.0
0.5
0.0
Percent of intensity attributed to semi-tryptic peptides
**
**
Untreated
PL 1.8 J/cm²
PL 7.1 J/cm²
Treatment
Untreated
PL 1.8 J/cm²
PL 7.1 J/cm²
* p<0.05
** p<0.01
*** p<0.001
C
Principal component analysis
15
10
5
0
−5
−10
−15
PC2(18.07%)
−15 −10 −5 0 5 10 15
PC1(49.01%)
Untreated
PL 1.8 J/cm²
PL 7.1 J/cm²
D
Protein significantly lower in abundance in the treated samples
log2(1.8/Untreated)
log2(7.1/untreated)
CotY
AsnO
MelA
PepA
SqhC
DacF
CotJA
CotSA
YqfQ
CotS
YloB
Tgl
CotI
CotG
YaaH
AtpA
CotH
YdhD
YjbG
LutC
SspN
YqfD
YoaN
CotB
YjqC
YqhO
PhoA
YhbB
YodI
YgaK
YfkD
DacB
Alr2
CotC
CotQ
SafA
SspO
CotA
YhxC
YabG
FtsH
PrsA
LipC
SodF
OppA
RpsK
CotE
YisY
YdcC
YhjR
CotF
CotR
YkuD
SpoVIF
YtxO
YkzQ
ResD
Mdh
RpsN1
log2(fold change)
-3
-2
-1
0


WT
cotG
spoVID
cotE
0
-1
-2
-3
-4
-5
-6
0
0.5
1
1.5
2
Reduction (log10)
Fluence (J/cm2)

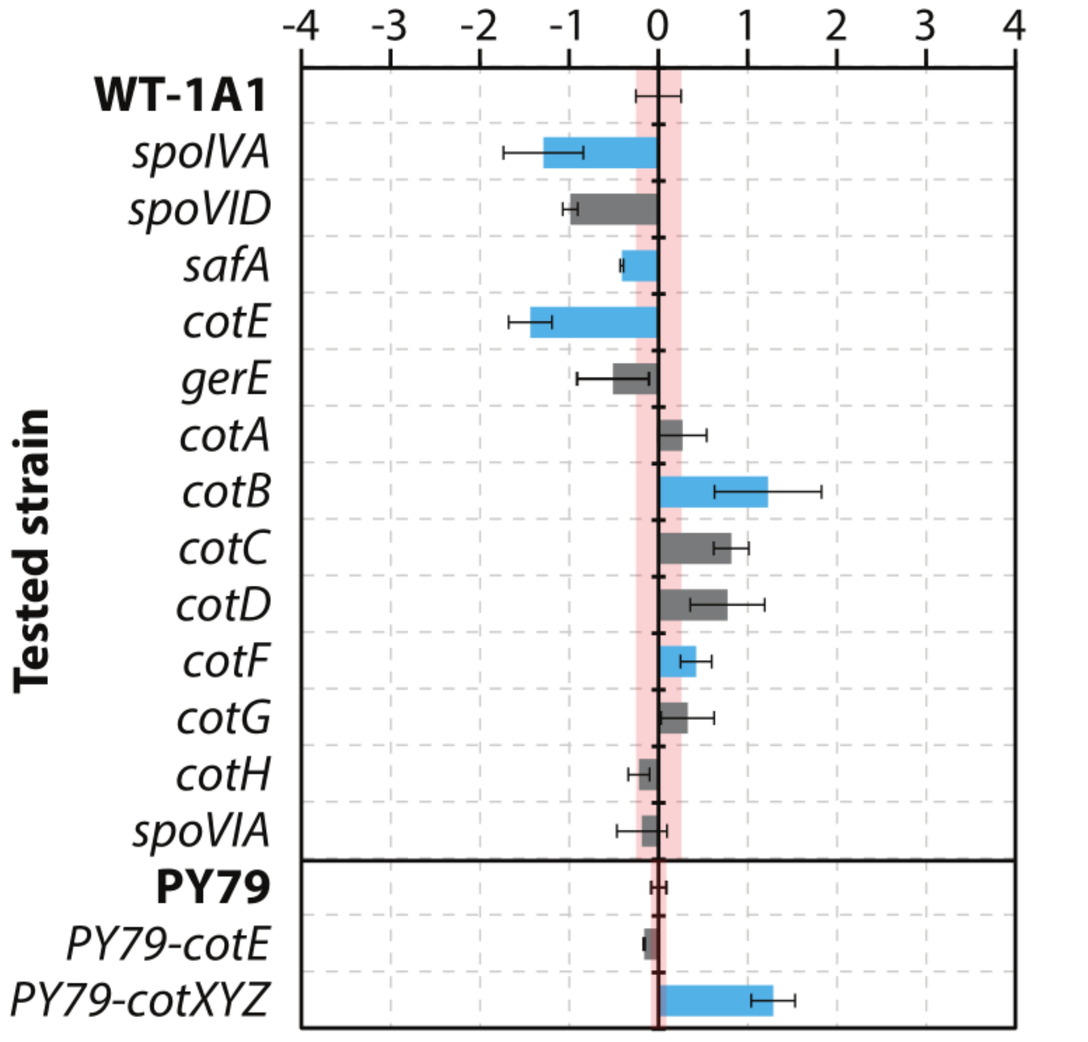
A

Pulsed Light Rmax (WT) - Rmax (tested strain)



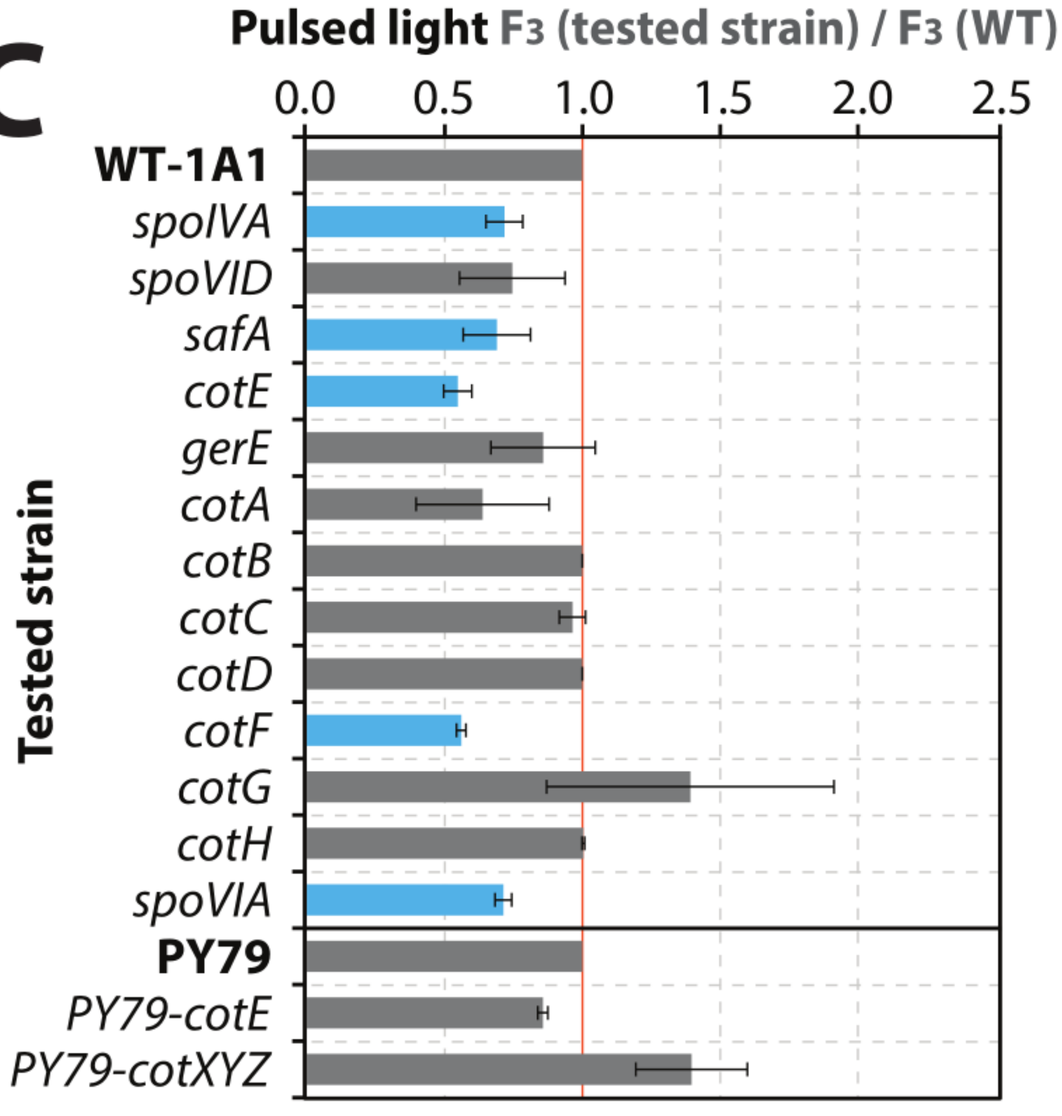
B

UV-C Rmax (WT) - Rmax (tested strain)



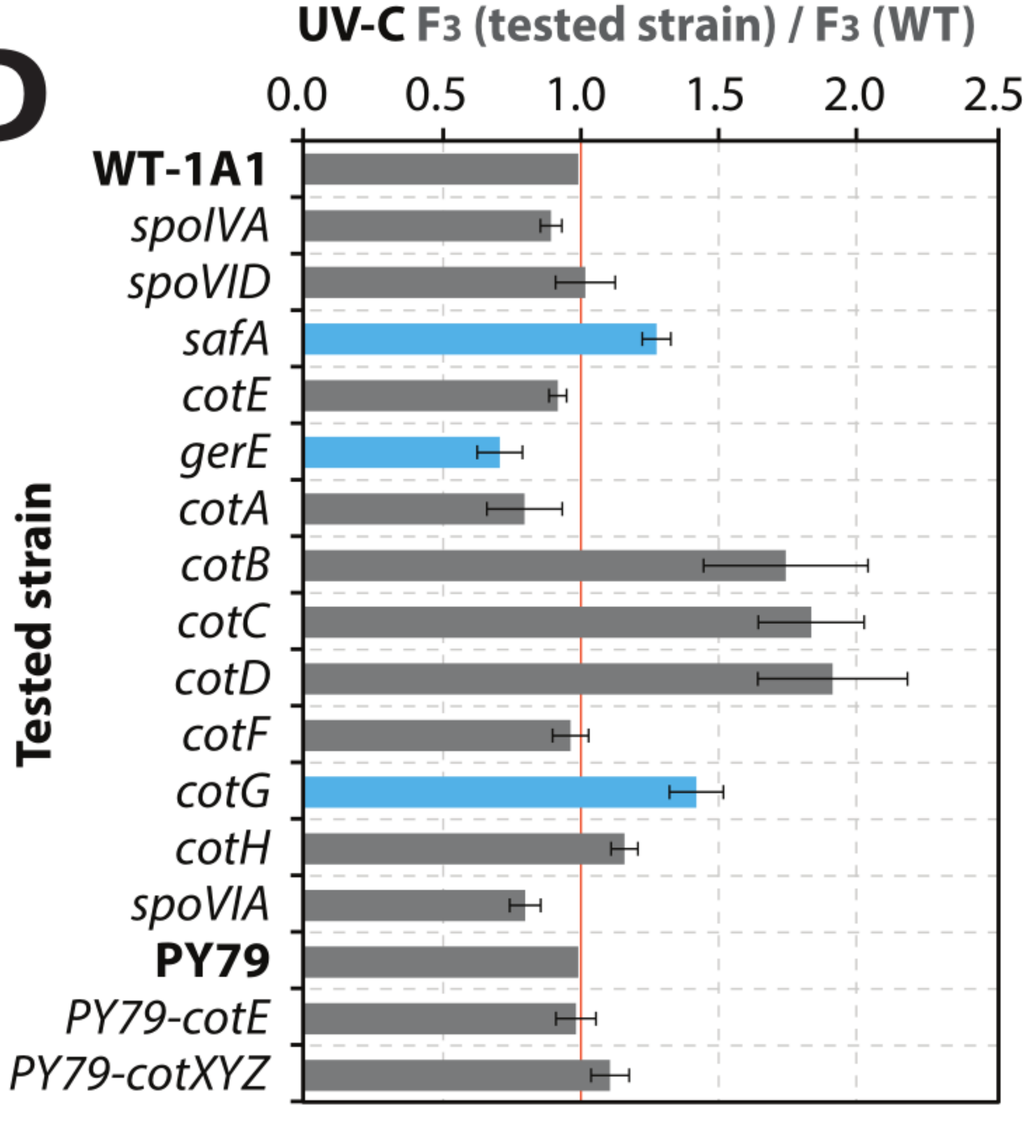
C

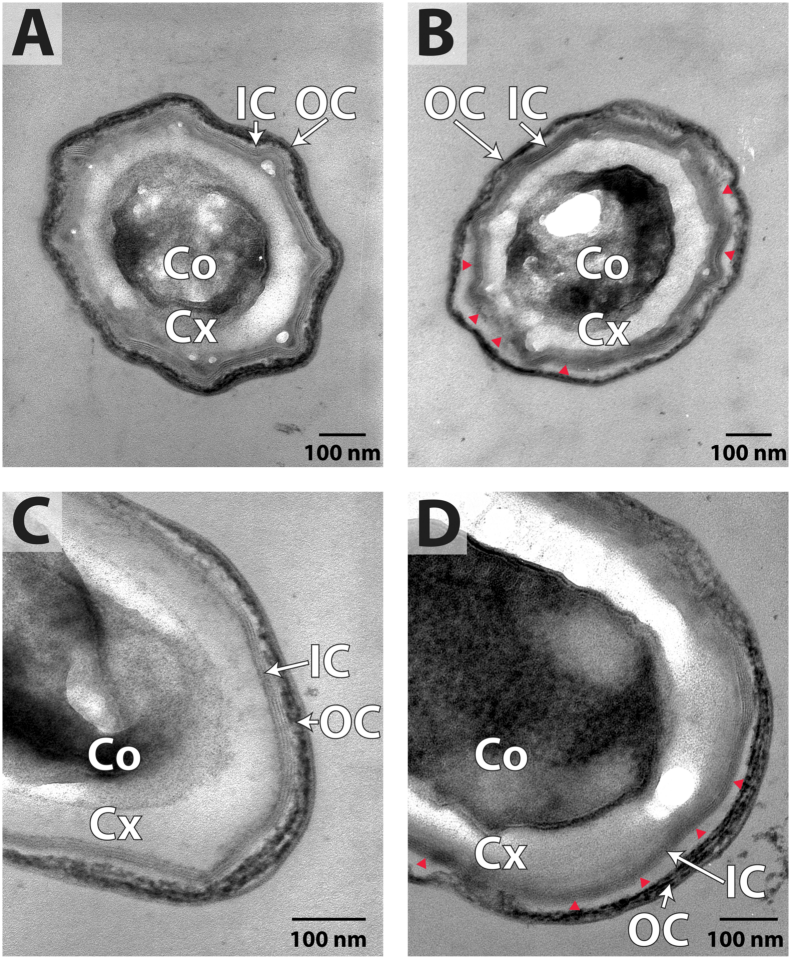
Pulsed light F3 (tested strain) / F3 (WT)



D

UV-C F3 (tested strain) / F3 (WT)




A
IC
OC
Co
Cx
100 nm
B
OC
IC
Co
Cx
100 nm
C
IC
OC
Co
Cx
100 nm
D
Co
Cx
IC
OC
100 nm