Directed evolution by UV-C treatment of *Bacillus cereus* spores

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Abstract

Bacterial endospores are exposed to a broad variety of sublethal and lethal stresses in the food production chain. Generally, these stresses will not completely eliminate the existing spore populations, and thus constitute a selection pressure on the spores. One stress that is frequently used in the food production chains to disinfect (food) contact surfaces is UV-C. At a wavelength of 254 nm, UV-C has germicidal properties. The aim of this research is to investigate the impact of UV-C stress on the evolution of endospore recalcitrance and germination in *B. cereus.*

A directed evolution experiment was set up in which *B. cereus* was repeatedly subjected to a cycle of sporulation, sporicidal UV-C treatment, germination and outgrowth. We show here that three independent lineages of UV-C cycled *B. cereus* spores reproducibly acquired a 30-fold or higher increase in UV-C resistance at 164 mJ/cm². Surprisingly, the UV-C resistant spores of the clones isolated from each of the lineages also became significantly more sensitive to wet heat as a normally non-lethal heat treatment at 70°C for 15 minutes resulted in an average 1.8 log cfu/mL reduction. From time-lapse phase contrast microscopy analysis, UV-C resistant mutant spores also showed a distinctive heterogeneity in refractility and a severe germination defect compared to the wild type. However, UV-C resistance of the corresponding vegetative cells were not altered.

In conclusion, this work shows that UV-C resistance of endospores is an adaptive trait that can readily be improved, although at an apparent cost for heat resistance and germination efficiency. As such, these results provide novel insights in the evolvability of, and correlation between, some endospore properties.

Key words: Endospore recalcitrance; UV resistance; heat resistance; germination defect; time-lapse microscopy; stress cycling

1. Introduction

Bacillus cereus endospores are very resistant to heat, dehydration and other physical stresses. They are able to survive in soil, sediments and dust (Nicholson et al., 2000; Stenfors Arnesen et al., 2008; Wassmann et al., 2012, 2010). As the spores of *B. cereus* are omnipresent in the environment, they find their way into the food processing plants and final food products (Carlin, 2011; Heyndrickx, 2011; Soni et al., 2016; Wells-Bennik et al., 2016). Upon germination and outgrowth some of the *B. cereus* strains can cause food spoilage or food poisoning, as most strains of *B. cereus* are toxin producers. *B. cereus* can cause two types of syndromes: (i) the emetic syndrome caused by the emetic toxin cereulide, or (ii) the diarrheal syndrome caused by one or more enterotoxins. At least three toxins are implicated in the diarrheal syndrome, namely haemolysin BL (Hbl), nonhaemolytic enterotoxin (Nhe) and cytotoxin K (CytK)(Fagerlund et al., 2008; Ganash et al., 2013; Lund et al., 2000; Stenfors Arnesen et al., 2008).

Food producers need to eliminate *B. cereus* or prevent their outgrowth to avert spoilage and foodborne disease. Bacterial endospores are exposed to a broad variety of sublethal and lethal stresses in the food production chain (Carlin, 2011; Heyndrickx, 2011; Stenfors Arnesen et al., 2008). Often, these stresses do not completely eliminate the existing spore populations, and thus constitute a selection pressure on the spores (Eijlander et al., 2011; Warda et al., 2015).

Endospores are highly resistant as their multi-layered endospore structure protects the DNA, ribosomes and spore enzymes, which can be found inside the core. The core has a low water content but is packed with the spore-specific molecule pyridine-2,6-dicarboxylic acid (dipicolonic acid, DPA). From the inside to the outside, the following layers can be found: core, inner spore membrane, germ cell wall, cortex, outer spore membrane, inner coat, outer coat, crust and –in some species- an exosporium. (Abhyankar et al., 2013; McKenney et al., 2013; Setlow, 2014; Thompson and Stewart, 2008).

The most common method used in the food industry to eliminate *B. cereus* is the application of heat. However, since heat has adverse effects on food quality the food industry is interested in using

nonthermal technologies such as UV-C (Bintsis et al., 2000; Coohill and Sagripanti, 2009; Gayán et al., 2013; Setlow, 2014). It must be noted that UV-C does not penetrate into foods and as a result its application for food treatment is limited, and it has more potential for treating food contact surfaces (Soni et al., 2016). At a wavelength of 254 nm, UV-C has germicidal and sporicidal properties (Bintsis et al., 2000; Coohill and Sagripanti, 2009; Gayán et al., 2013; Setlow, 2014). The primary target of UV-C damage in microbial cells is DNA (Moeller et al., 2014; Setlow, 2014).

Spores are 20-50 times more resistant to UV than their vegetative counterparts (Setlow, 2014). This is due to the fact that spores have: (i) a change in DNA's UV photochemistry due to binding of α/β-type Small, acid-soluble spore proteins (SASP), (ii) dedicated DNA repair mechanisms, (iii) pigments in the outer layer of some spores. A factor that negatively influences the spore's UV-C resistance is the DPA depot, since spore that lack DPA are more resistant to UV-C (Paidhungat et al., 2000; Setlow, 2006). In vegetative cells, UV-C at a wavelength of 254 nm generates photoproducts between adjacent pyrimidines in the same DNA strand: 6,4-photoproducts or cyclobutane dimers. These photoproducts are potentially lethal. In contrast, UV radiation of spores generates a thyminyl-thymine adduct named spore photoproduct (SP). This SP is much less lethal than the photoproduct formed in vegetative cells. The reason why a different photoproduct is formed in spores is that their DNA is saturated with α/β SASPs that change the DNA structure from a B-conformation to something between A and B –DNA. This results in a changed DNA photochemistry and a higher resistance to UV-C (Mason and Setlow, 1986; Setlow, 2014, 2006).

Directed (laboratory) evolution approaches are becoming increasingly fruitful in food microbiology research to examine the adaptive potential of foodborne pathogens and spoilage organisms (Elena and Lenski, 2003; Gayán et al., 2016; Vanlint et al., 2012; Zeigler and Nicholson, 2017). In contrast to the evolution of vegetative cell properties, the impact of the selection pressure on the evolution and properties of spores has not yet been addressed. In order to investigate the impact of UV-C stress on the evolution of endospore recalcitrance in *B. cereus*, a directed evolution experiment was set up in

which *B. cereus* was repeatedly subjected to sporulation, UV-C treatment, germination and outgrowth. We show here that iterative cycles of UV-C stress on spores of *B. cereus* result in *B. cereus* spores with considerably increased UV-C resistance.

2. Material & methods

2.1. Strains and spore production

Stock cultures of ATCC 14579 and resulting controls and clones were stored at -75◦C in Brain Heart Infusion (BHI, Oxoid, Basingstoke, England), supplemented with 10% or 25% glycerol (Prolabo, Heverlee, Belgium). Working stocks were stored refrigerated at 4◦C on BHI agar and were renewed monthly. The sporulation protocol was based on Garcia et al. (Garcia et al., 2010). In short, a single colony was transferred initially into BHI (Oxoid) and incubated at 30◦C for 18 to 24 h. 0.5 mL of this overnight culture was transferred into 50 mL Maltose sporulation medium (1mM $MgCl_2.6H_2O$, 1mM $Ca(NO_3)_2.4H_2O$, 12.5 µM $CuCl_2.2H_2O$, 12.5 µM $ZnCl_2$, 2.5 µM $CoCl_2.6H_2O$, 2.5 µM $Na_2MoO_4.2H_2O$, 5mM $(NH_4)_2SO_4$, 66 µM $MnSO_4.H_2O$, 10mM maltose, 1µM $FeSO_4$) and incubated shaken at 250 RPM for 72 h (van der Voort et al., 2010). Spores were washed three times with 100mM phosphate buffer supplemented with 0.1% Tween 80, incubated for one hour in 50% ethanol and washed three times with 100mM phosphate buffer with 0.01% Tween 80.

2.2. Directed evolution of endospores for UV-C resistance

Five independent batches of spores were produced from five different colonies of a *B. cereus* ATCC 14579 stock plate as a starting point. Three batches of these spores were subjected to five iterative cycles of sporulation, sporicidal UV-C treatment, germination and outgrowth (referred to as stressed-lineages; Fig. 1), while two control batches were cycled without stress (producing new generation of spores starting from 100-10,000 spores/mL; referred to as unstressed-lineages; Fig. 1) to determine the effect of repetitive sporulation, germination, and outgrowth. In addition, wild type spores were freshly sporulated at each cycle to check the parental level resistance to corresponding UV-C treatment (referred to as parental control) The lineages were sporulated in maltose sporulation

medium (see 2.1) for 72 hours and washed, diluted tenfold in 100mM phosphate buffer with 0.01% Tween80 and subjected to a UV-C treatment (see 2.3) to obtain a targeted reduction of 2.5 ± 0.5 log cfu/mL reduction. Next, the UV-C treated spores were diluted in Peptone Physiological Solution (PPS) (1 g/L pepton (Oxiod, Oxford, England) + 8.5 g/L NaCl (Sigma-Aldrich, St. Louis, USA)) with 0.01% Tween 80 and 3.0 ± 1.0 log cfu/mL was transferred to a new batch of maltose sporulation medium. The unstressed lineages were also washed in 100mM phosphate buffer with 0.01% Tween80. They were diluted 1000x in PPS before inoculated in fresh MSM medium for outgrowth and sporulation.

2.3. UV-C treatment of spores and vegetative cells

During the cycling experiment and the UV-C resistance confirmation experiments, washed spores were 10x diluted upon UV-C treatment in a 100mM phosphate buffer (pH 7.4) supplemented with 0.01% Tween 80 and 8mL was transferred to a Petri dish (55mm, Gosselin Plastics, Borre, France) with a magnetic stir bar. The spores were continuously stirred during the UV-C treatment on a magnetic stirrer (setting 5, RCT basic, IKA, China). UV-C treatment was performed in a closed inox box with a UVpro K17-2 lamp (BioClimatic, The Netherlands). The UV-C dose was increased per cycle starting from 96 mJ/cm² to 164 mJ/cm². Dosimetry was the average of 3 measurements with an ILT 1700 radiometer connected to XRD140T254 detector (International Light technologies, Peabody, USA). During the cycling experiment, the initial spore concentration of stressed lineages and parental was enumerated after a heat treatment of 70°C for 15 minutes to remove vegetative cells or germinated spores. In cycle 5 the intensity of this heat treatment was reduced to 60°C for 15 minutes.

For the UV-C inactivation of vegetative cells from UV-C cycled spores, the clones were subcultured twice in BHI for 24h at 30°C. The vegetative cells were diluted to a target concentration of 3 log cfu/mL and incubated during 5 hours at 30°C to reach the mid-exponential phase. After 5 hours the samples were diluted 10x in PPS with 0.01% Tween80 to a target concentration of 5 log cfu/mL. Similar to the UV-C treatment of spores, 8 mL of samples was placed in a Petri dish (55mm, Gosselin

Plastics, Borre, France) with magnetic stir bar. In addition, an extra metal plate was placed inside the UV-C chamber between the UV lamp and the sample to reduce the radiated surface to 1 cm^2. After radiation, the samples were diluted in PPS with 0.01% Tween 80, then plated on BHI agar and incubated at 30°C for 48 hours. Every dilution series was performed twice and experiments were performed in triplicate. Using the excel tool, Ginafit, a log-linear curve was fitted on the first three time points of the inactivation curve (Geeraerd et al., 2005).

2.4. Wet heat treatment

To check for cross-resistance against wet heat stress, 50µL of spores suspensions were transferred to Sterile Ultra-Thin (UTW) walled PCR tubes (0.2mL; BIOplastics, Landgraaf, The Netherlands). Wet heat treatment was done in a PCR cycler (Artktik Thermal cycler – Thermo Scientific, Waltham, USA) with the following settings: 105°C lid temperature, sample temperature at 70°C, 15 minutes of treatment time and then cooling down to 4°C. At defined time points, samples were taken out and place in an ice bath. Spores were enumerated as explained in 2.5.

2.5. Enumeration of spores

Preliminary experiments showed no difference in spore counts with or without a heat shock of 80°C for 10 minutes or 70°C for 15 minutes (data not shown). All samples were tenfold diluted in PPS (1 g/L pepton (Oxiod, Oxford, England) + 8.5 g/L NaCl (Sigma-Aldrich, St. Louis, USA)) with 0.01% Tween 80 and plated on BHI supplemented with 1.5 % agar (Agar Bacteriological, Oxoid, Oxford, England). Surviving spores were counted after 72 hours incubation at 30°C.

2.6. Spore count determination with Thoma counting chamber

Due to germination defects in spores from stressed lineages, actual spore count was determined using phase contrast microscope (Leica, Germany) with Thoma counting chamber (Paul Marienfield, Germany). Three independent spore stocks of wild type and first isolate of stressed lineage A (clone A1, as a representative of stressed lineages) were counted.

2.7. Quality control during cycling experiment

To confirm that no environmental or lab related contamination occurred, PCR and agarose gel-electrophoresis were performed on isolates at certain time points during the cycling experiment to confirm that the spores were all descendants from the parental ATCC 14579 strain. The first primer pair (BcM_BC5267F1 and BcM_BC5267R1) is specific for *B. cereus sensu stricto* strain ATCC 14579 and targets the BC5267 gene. The wild type (parental) and all the cycled lineages (including UV-C treated and non- UV-C treated lineages)should test positive for this primer pair. In addition, two other primer pairs were included in this quality control. The target genes of these primer pairs are not present in ATCC 14759 and as a results the PCR results should be negative for all lineages. DNA was extracted from vegetative cells by heating the samples in a lysis buffer (10 % sodium dodecylsulfate, 1N NaOH). PCR was performed with the Q5™ High-Fidelity 2X Master Mix (New England Biolabs inc, Massachusetts, USA) and the following primers were used: BcM_BC5267F1 5'-CCAAAGAGTTAAGGGCTGTTAG-3', BcM_BC5267R1: 5'-GTTGAAGGGTTCTTACCAAGATG-3', BcT_cytK1_F: 5'-TACCAGTTCCGAATGTAAAGC-3', BcT_cytK1_R: 5'-GCTTAGTCATCGCTGGTG-3', BcP_cspA_F: 5'-TCGAAGTTCCAGGCGAAAAC-3' and BcP_cspA_R: 5'-CTGTTCTTCACGAAGCCTTTTC-3'. Only the primers BcM_BC5267F1 and BcM_BC5267R1 are specific for the parental strain and show a band at 680 bp on gel (data not shown).

2.8. Germination assays

Spores of the UV resistant and control clones were germinated in an assay containing a high and a low concentration of the germinant L-alanine (Acros Organics, Geel) (Broussolle et al., 2008).

The spores were centrifuged at 10 000 xG for 15 minutes at 4°C and suspended in a germination buffer of 10mM Tris/HCl (Trizma base (Sigma-Aldrich, St. Louis, USA) + HCl (Sigma-Aldrich, St. Louis, USA)) (pH = 7,4) and 20mM D-cycloserine (Sigma-Aldrich, St. Louis, USA) supplemented with 0.01% Tween 80 after the supernatant was removed. D-cycloserine was added to prevent the alanine racemase activity of spores (Broussolle et al., 2008).

Germination was monitored by measuring the optical density decrease at 600 nm in a Versamax microplate reader (Molecular Devices, San Jose, USA) during six hours incubation at 30°C. The OD_{600} was read every 2 minutes after firmly shaking the microplate to prevent settling of spores. The percentage of the OD_{600} decrease was calculated using following formula: $[1 - \{OD_t - (OD_{Ct} - OD_{C0})\} / OD_0] \times 100$, where OD_t and OD_{Ct} are the OD_{600} values for respectively the test sample and negative control (buffer without L-alanine, but with spores) measured after time t, and where OD_0 and OD_{C0} are the initial OD_{600} values for respectively the test sample and negative control. This percentage expressed the extent of germination (Broussolle et al., 2008).

2.9. Time-lapse phase contrast microscopy (TLM)

To quantify different subpopulations in spore stocks and germinating spores among them, time-lapse phase contrast microscopy analysis was performed with an Eclipse Ti-E inverted microscope (Nikon Instruments Europe BV, Netherlands) equipped with a microscope incubator (Okolab, Italy) and a TI-CT-E motorized condenser. Images were acquired by CoolSnap HQ2 FireWire CCD camera using NIS Element (Nikon), and were further handled with open source software FIJI (Schindelin et al., 2012). For Imaging of *B. cereus* spores and their germination, spores were placed between agarose pads and a cover glass using Gene Frames (Thermo Fischer Scientific, USA). However, the germination response of *B. cereus* spores to nutrients is very rapid, and spores already turn phase dark during the preparations of agarose pad and microscopy. To overcome this issue, a nutrient diffusion time-lapse microscopy technique was applied. In short, a 4X BHI + 1.5% agarose pad is positioned next to a pure agarose pad (1.5% agarose in sterile miliQ water) on a glass slide and it is pushed towards the pure agarose pad to make contact just before placing the slide on the microscope platform, so that nutrients start diffusing into the pure agarose pad. In this way, the germination response can be delayed enough to capture the initial state of the spores in the middle part of the pure agarose pad. More than 2,000 spores were counted from each spore stock of three independent spore productions of wild type and the first isolate from stressed lineage A.

2.10. Statistical analysis

All microbial resistance determinations were performed at least 3 times on different working days. Statistical analyses, boxplots to detect outliers, t-test ($p = 0.05$), and ANOVA tests ($p = 0.05$) were carried out using the SPSS software version 25 (IBM SPSS Statistics for Windows, New York, USA), and differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Directed evolution for UV-C resistance

The results of the directed evolution with UV-C on *B. cereus* (ATCC 14759) endospores are displayed in figure 2. The UV-C dose varied from cycle to cycle. For the first cycle the UV-C dose was 96 mJ/cm², to ensure the targeted 2-3 log cfu endospores/mL reduction this was increased to 107 mJ/cm² for the next 3 cycles and finally a UV-C dose of 164 mJ/cm² was applied in the last cycle. After four cycles, two out of three UV-C stressed lineages showed remarkable increase in UV-C resistance. The fifth and last cycle had an increased dosage of 164 mJ/cm² and at these dosage all three lineages showed no endospore reduction, while the parental control showed ca. 4 log cfu/mL reduction. These results demonstrate that directed evolution can significantly increase the UV-C resistance of *B. cereus* endospores. All lineages are confirmed as descendants from ATCC 14759 with PCR and gel-electrophoresis (data not shown). To determine that only spores were counted, the aliquot used to count the spores before UV-C treatment received a heat shock of 70°C for 15 minutes to eliminate vegetative cells or germinated spores. In cycle 5 the initial spore counts before UV-C treatment decreased, even to lower numbers than the counts after UV-C treatment (panel B, Fig. 2). This implicates a possible inactivation during the heat treatment step and a milder heat treatment at 60°C for 15 minutes yielded a higher initial spore count. This rules out the possibility of vegetative cells being responsible for this increase and it was the first hint linking UV-C resistance to wet heat sensitivity.

Per lineage, three random clones were purified and UV-C resistance of the corresponding spore crops was confirmed with a dosage of 164 mJ/cm² (Fig. 3). All stress-cycled isolates, with the exception of isolate two in lineage three, were confirmed to have significantly increased UV-C resistance ($p<0.05$) compared to the parental control. Non-stressed cycled lineages did not acquire significant UV-C resistance, indicating that cycling alone does not select for this phenotype. On average all UV cycled isolates had a reduction of 1.1 ± 0.8 log cfu/mL, compared to 3.1 ± 0.6 log cfu/mL reduction for wild type and non-stress cycled isolates. More in detail, the average reduction for isolate A1 is 0.9 ± 0.4 log cfu/mL, isolate A2 0.6 ± 0.4 log cfu/mL, isolate A3 0.8 ± 0.6 log cfu/mL, isolate B1 0.8 ± 0.6 log cfu/mL, isolate B2 0.8 ± 0.9 log cfu/mL, isolate B3 1.0 ± 0.5 log cfu/mL, isolate C1 1.3 ± 0.8 log cfu/mL, isolate C2 2.2 ± 1.1 log cfu/mL and isolate C3 1.2 ± 0.6 log cfu/mL.

3.2. Heat resistance of UV-C resistant clones

To examine to what extent acquisition of UV-C resistance could coincide with cross-resistance to other stresses such as heat, spore crops of clones A1, B1, C1, X1 and Y1 where tested against wet heat inactivation at 70°C (Fig. 4). Surprisingly, UV-C resistant clones proved to be heat sensitive, and displayed viability reduction within the first minute of respectively 1.1 ± 0.3, 1.2± 0.2 and 1.4 ± 0.1 log CFU/mL for clone A1, B1 and C1. The non-stress cycled (X1 and Y1) and parental control showed no significant reduction. After the decrease in the first minute a tail is visible with a reduction over the next 14 minutes of respectively 0.7 ± 0.2, 0.6± 0.2 and 0.3 ± 0.2 log CFU/mL for isolate A1, B1 and C1. These results indicate that there is a subpopulation sensitive to wet heat, possibly as a trade-off for UV-C resistance.

3.3. Germination defect in UV-C resistant clones

From preliminary phase contrast microscopy, the total number of spores of UV-C resistant clones in microscopic fields was similar to that of the wild type. However, the clones showed decreased cfu on BHI plate count of 8.5 ± 0.2 log CFU/ml, while the wild type showed 9.8 ± 0.1 log CFU/ml (Table 1). A germination assay employing optical density (OD) at 600 nm proved that the addition of the

247 germinant L-alanine, either in high or in low concentration, did not result in a noticeable OD drop for
248 spore crops of the UV-C resistant clones (Fig. 5). In contrast, the parental control and non-stressed
249 cycled controls (clone X1 and Y1) did display an OD decrease. For the parental control the OD
250 decrease one hour after the addition of 100 mM L-alanine was 26.0 % compared to 7.8 % for 10 mM
251 L-alanine. For non-stress cycled controls (X1 and Y1) and the parental control the mean percentage
252 of OD decrease was respectively 51, 52 and 51 % after four hours of incubation in 10 mM L-alanine
253 and respectively 56, 55 and 56 % after four hours of incubation in 100 mM L-alanine. These results
254 indicate that a substantial germination defect exists in spores of the UV-C resistant clones.

255 To determine the accurate spore concentrations, a Thoma counting chamber was used (Table 1) for
256 three independent spore stocks of the wild type ATCC 14579 and clone A1. As all independently
257 evolved lineages showed similar phenotypes, the clone A1 was used in further analyses as a
258 representative of UV-C resistant clones. As seen in Table 1, actual spore concentration of A1 from
259 Thoma counting chamber was comparable to the wild type. The lower plate count of clone A1
260 compared to the wild type can be explained by a lower germination frequency. Time-lapse phase
261 contrast microscopy was also performed to see how many spores actually responded to nutrient-
262 induced germination by BHI medium. Using both the plate count/Thoma counting chamber ratio and
263 time-lapse microscopy observations, it was estimated that approximately only 5% of the spores
264 germinated in the A1 clone (Table 1).

265 Another distinctive difference between the wild type and the obtained UV-C resistant clones is spore
266 morphology (Fig. 6, lower panel). Indeed, the refractility within UV-C resistant spore crops was more
267 heterogeneous compared to the parental spore population. It is important to highlight here that the
268 heterogeneity in refractility has an important impact on the results in the OD germination assay (Fig.
269 5). The bulk OD measurements of Fig. 5 would not be able detect possible germination events
270 stemming from the already phase-dark spores. However, a much more detailed subsequent TLM
271 analysis clearly confirms the overall germination defect, and furthermore indicates that the group of

phase-dark/grey spores displays very low germination efficiency (Fig. 6).A substantial amount of phase dark and grey spores was also found in spore stocks of A1 clone while over 95% of spores were phase bright in the wild type (Fig. 6, top panel). Figure 6 shows which type of spores were germinated under TLM. Small fractions of phase dark and grey spores in A1 were able to germinate while only phase bright spores were germinated in the wild type. This might indicate that the phase dark spores in A1 are different from those of the wild type in terms of viability.

3.4. UV-C inactivation of vegetative cells of UV-C cycled spores

As discussed previously in 3.1, spores of all three UV-C cycled lineages and corresponding spore crops of clones A1, B1 and C1 showed an increased resistance against UV-C, compared to the wild type. To verify if this increased resistance also affects the UV-C resistance of their vegetative counterparts, triplicate experiments were performed to assess the UV-C inactivation of the corresponding vegetative cells in liquid. The vegetative cells displayed a two-phase inactivation curve and there were no differences found in inactivation between A1, B1, C1 clones and the wild type (Fig.7). Log-linear inactivation curves, using the first three time points, were fitted using GInafit. The inactivation rate (K_{max}) ± SE is 1.46 ± 0.28 for A1, 1.24 ± 0.18 for B1, 1.54 ± 0.19 for C1 and 1.54 ± 0.25 for the wild type. Our results show that although the UV-C cycled spores are more resistant to UV-C, this trait is not present in their vegetative stage.

4. Discussion

Directed evolution experiments with UV-C inactivation help to understand the mechanisms and evolvability of endospore recalcitrance. While directed evolution experiments with UV-C stress on vegetative cells have been reported in literature, this is to the best of our knowledge the first directed evolution approach on UV-C resistance on bacterial endospores. We show here that repeated exposure of *B. cereus* endospores to UV-C stress reproducibly results in the selection of mutants yielding spores with an increased UV-C resistance (≥ 30 fold compared to the wild type at 164 mJ/cm²).

297 *B. cereus* does not only have the ability to spoil food, it can also produce toxins and cause illness. In
298 the food industry, UV is applied to disinfect (food) contact surfaces, water and air (Bintsis et al.,
299 2000). Taken into account the relatively short time it took to get UV-C resistance, an in-house
300 microbiota of *B. cereus* spores could potentially get increased resistance towards the UV-C
301 disinfection techniques used. We show here that three independent lineages acquired UV-C
302 resistance. However, this resistance is accompanied by compromised heat resistance and
303 germination deficiencies and this will lower the overall survival capacities of these spores in food
304 processing. Another application for this research is in the biopesticides industry. *B. thuringiensis* has
305 been used for many years as a biopesticide. Multiple research focusses on increasing the resistance
306 of *B. thuringiensis* to UV and thus prolonging the persistence on the field (Saxena et al., 2002; Zhang
307 et al., 2018, 2016).

308 Interestingly, it seems that all three independently evolved lineages display similar phenotypes in
309 which an increased UV-C resistance was inevitably accompanied by a decreased wet heat resistance
310 and germination efficiency. With regard to the reduced germination efficiency, microscopic analysis
311 indicates that UV-C resistant spore crops display an increased fraction of phase dark and grey spores.
312 However, this increased fraction cannot solely be responsible for the wet heat sensitivity as the
313 reduction found after wet heat treatment was bigger than the fraction phase-dark and phase-grey
314 spores. It was recently suggested for *B. subtilis* that defects in spore coat assembly or core
315 dehydration during late sporulation stage may lead to premature germination triggered by GerA,
316 eventually yielding phase dark or grey spores (Ramírez-Guadiana et al., 2017). In addition, the
317 lipoproteins, YlaJ and YhcN have been shown to contribute to the efficiency of spore germination in
318 *B. subtilis* (Johnson and Moir, 2017). It is known that low DPA levels increases the resistance of
319 spores to UV-C, increases core water activity and sensitizes spores to wet heat stress. Notably, spores
320 with low DPA levels tent to germinate spontaneously (Paidhungat et al., 2000). However, since UV-C
321 resistance, heat resistance and germination are supported by complex multifactorial processes
322 (Setlow, 2014, 2006), their interrelationship will be further addressed in the future.

323 The UV-C resistance of the vegetative cells was not affected by our evolution. This is in agreement
324 with the fact that the damage inflicted by UV-C is different between spores and vegetative cells. UV
325 radiation of spores produces the thyminyl-thymine adduct that is named spore photoproduct (SP)
326 and that is much less lethal than the pyrimidine dimers formed in vegetative cells (Setlow, 2014).

327 This work provides useful insights in endospore evolution and its impact on the surviving *B. cereus*
328 spores. Directed evolution of *B. cereus* spores by UV-C could drive several independent lineages
329 within a relatively short time to acquire increased UV-C spore resistance while trading off wet heat
330 resistance and germination efficiency.

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Tables

Table 1. Spore concentration and germination rate of the wild type and UV resistant clone A1.

	Spore concentration (log CFU/ml)		**% Germination**	
	Thoma chamber	Plate count	Plate/Thoma	TLM*
WT	9.8 ± 0.1	9.8 ± 0.1	94.5 ± 15.6%	94.5 ± 0.8%
A1**	9.8 ± 0.1	8.5 ± 0.2	5.4 ± 2.8%	4.9 ± 1.0%

TLM*: Data from nutrient diffusion time-lapse phase contrast microscopy.

A1**: Isolate A from stressed lineage 1 as a representative of resistant mutants.

Figure captions

Figure 1. Set-up of cycling experiment. Three lineages were started independently from each other from a single colony of *Bacillus cereus* ATCC 14579. Each lineage (lineage A, B, and C) underwent subsequent cycles of sporulation, UV-C treatment to target 2-3 log cfu/mL reduction, germination and growth. Two types of controls were conducted: (i) two control lineages cycled without stress (unstressed lineages, lines X and Y) and (ii) one control wild type spore stock of ATCC 14579 freshly sporulated at each cycle to check initial UV-C resistance level of ancestral population.

Figure 2. Results of directed evolution with UV-C stress on B. cereus endospores. (A) Three independent lineages were cycled for UV-C stress (lineage A, B and C). UV-C reduction in log N/N_0 are displayed. For log N_0 (spore counts prior to UV-C treatment, in log cfu/mL) the spore solution received a heat shock of 70°C for 15 minutes before plating. UV-C dosage is displayed on top of the graph. (B) Detailed overview of the spore concentrations (log N_0) in cycle 5 of the directed evolution experiment. Spore were counted after no heat treatment, 70°C for 15 minutes or 60°C for 15 minutes.

Figure 3. Confirmation of UV-C resistance of clones isolated from the cycling experiment. Three clones were isolated per lineage. All UV-C resistant isolates – except isolate 2 of lineage C- are significantly more resistant to UV-C at a dose op 164 mJ/cm² compare to the wild type. * Indicates significant difference ($p<0,05$) compared to control group evolved without UV treatment and wild type.

Figure 4. Heat resistance of selected UV-C clones at 70°C. UV- resistant isolate A1, isolate B1 and isolate C1 was compared to the non-stress controls (isolate X1 and isolate Y1) and the wild type at 70°C for 15 minutes. Mean plate counts of 5 replicates are displayed. Out of five replicates per isolate extreme outlier were detected with boxplots and removed from the statistical analysis.

Figure 5. Germination assays with L-alanine. Mean percentages of optical density drop at 600 nm of clone A1 (▲), clone B1 (●) and clone C1 (◆) and control X1 (▲), control Y1 (●) and wild type (◆)

with (A) 10 mM and (B) 100mM L-alanine in function of time. Means of four biological replicates and standard deviation are displayed.

Figure 6. Germination time-lapse microscopy results in detail. Left side panel is the wild type and A1 is on the right. Top panel: total number of analyzed spores and a representative image of the spore crop. Middle: subpopulation composition based on relative refractility and the number of spores in each category. Bottom: germination efficiency and the number of germinated spores in each category.

Figure 7. UV-C inactivation of vegetative cells of UV-C cycled spores and the wild type. Three biological replicate were performed and mean plate counts are displayed.

Figure 1

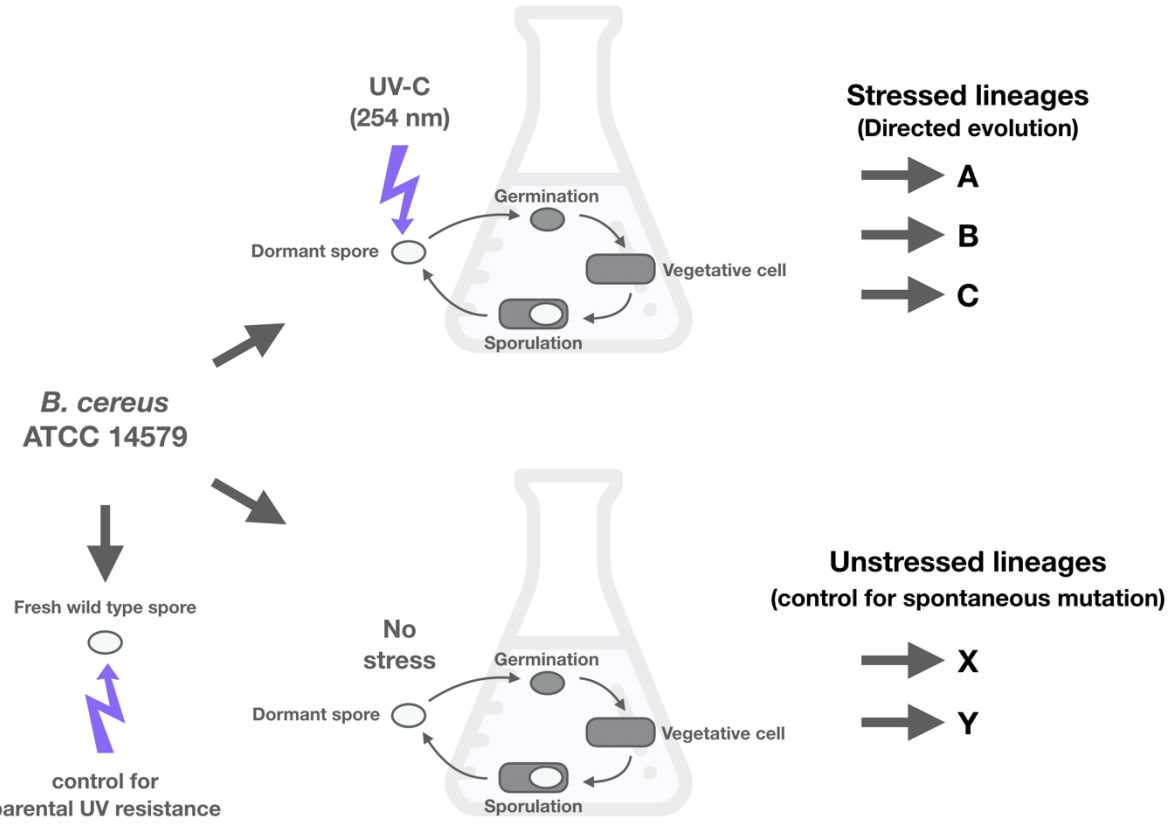

UV-C
(254 nm)
Germination
Dormant spore
Vegetative cell
Sporulation
Stressed lineages
(Directed evolution)
A
B
C
B. cereus
ATCC 14579
Fresh wild type spore
control for
parental UV resistance
No
stress
Germination
Dormant spore
Vegetative cell
Sporulation
Unstressed lineages
(control for spontaneous mutation)
X
Y

Figure 2

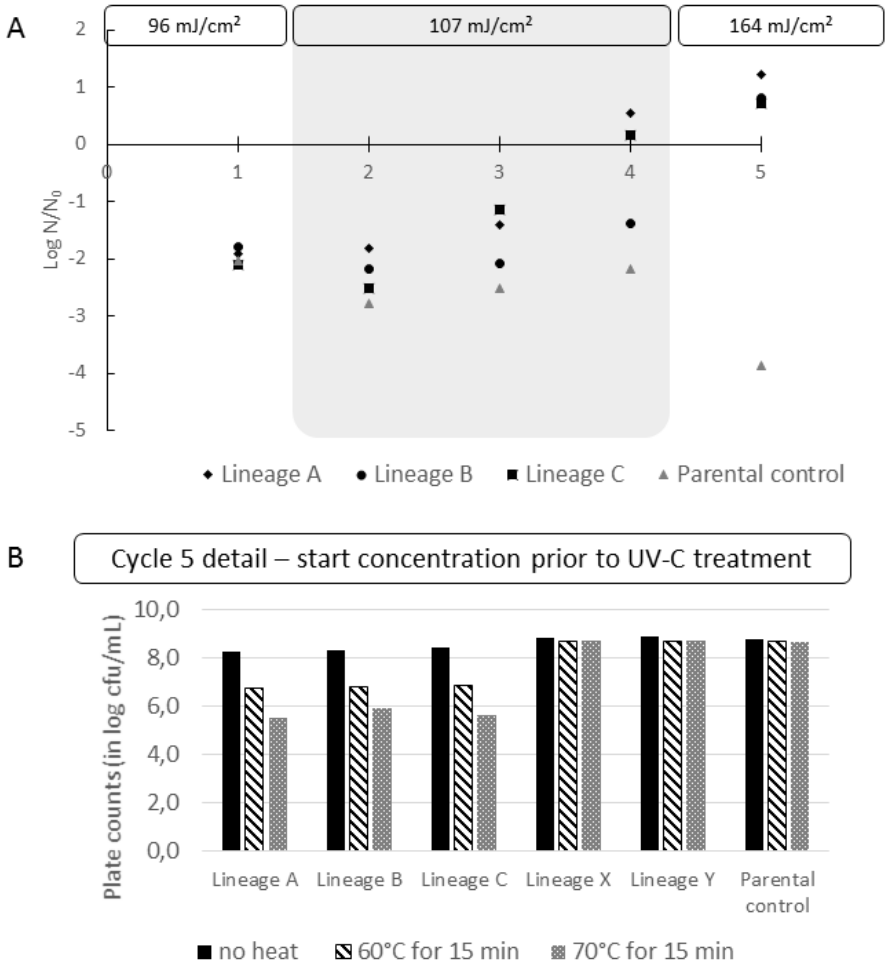

A
96 mJ/cm²
107 mJ/cm²
164 mJ/cm²
Log N/N0
Lineage A
Lineage B
Lineage C
Parental control
B
Cycle 5 detail – start concentration prior to UV-C treatment
Plate counts (in log cfu/mL)
Lineage A
Lineage B
Lineage C
Lineage X
Lineage Y
Parental control
no heat
60°C for 15 min
70°C for 15 min

Figure 3

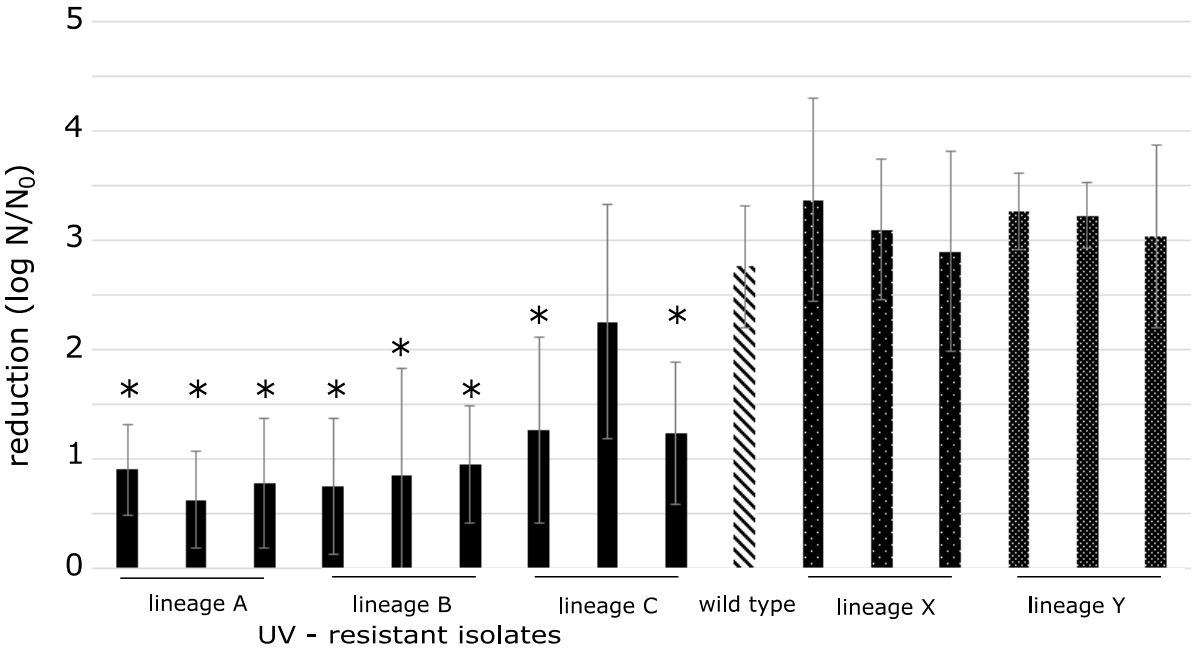

5
4
3
2
1
0
reduction (log N/N0)
*
*
*
*
*
*
*
*
lineage A
lineage B
lineage C
wild type
lineage X
lineage Y
UV - resistant isolates

Figure 4

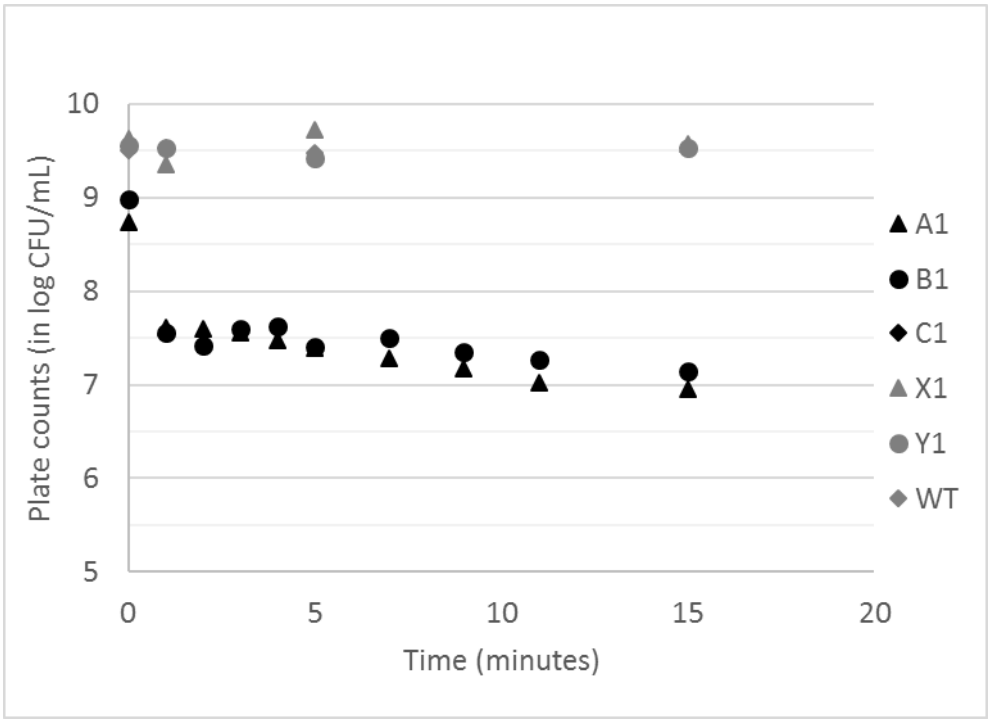

10
9
8
7
6
5
Plate counts (in log CFU/mL)
0
5
10
15
20
Time (minutes)
A1
B1
C1
X1
Y1
WT

Figure 5

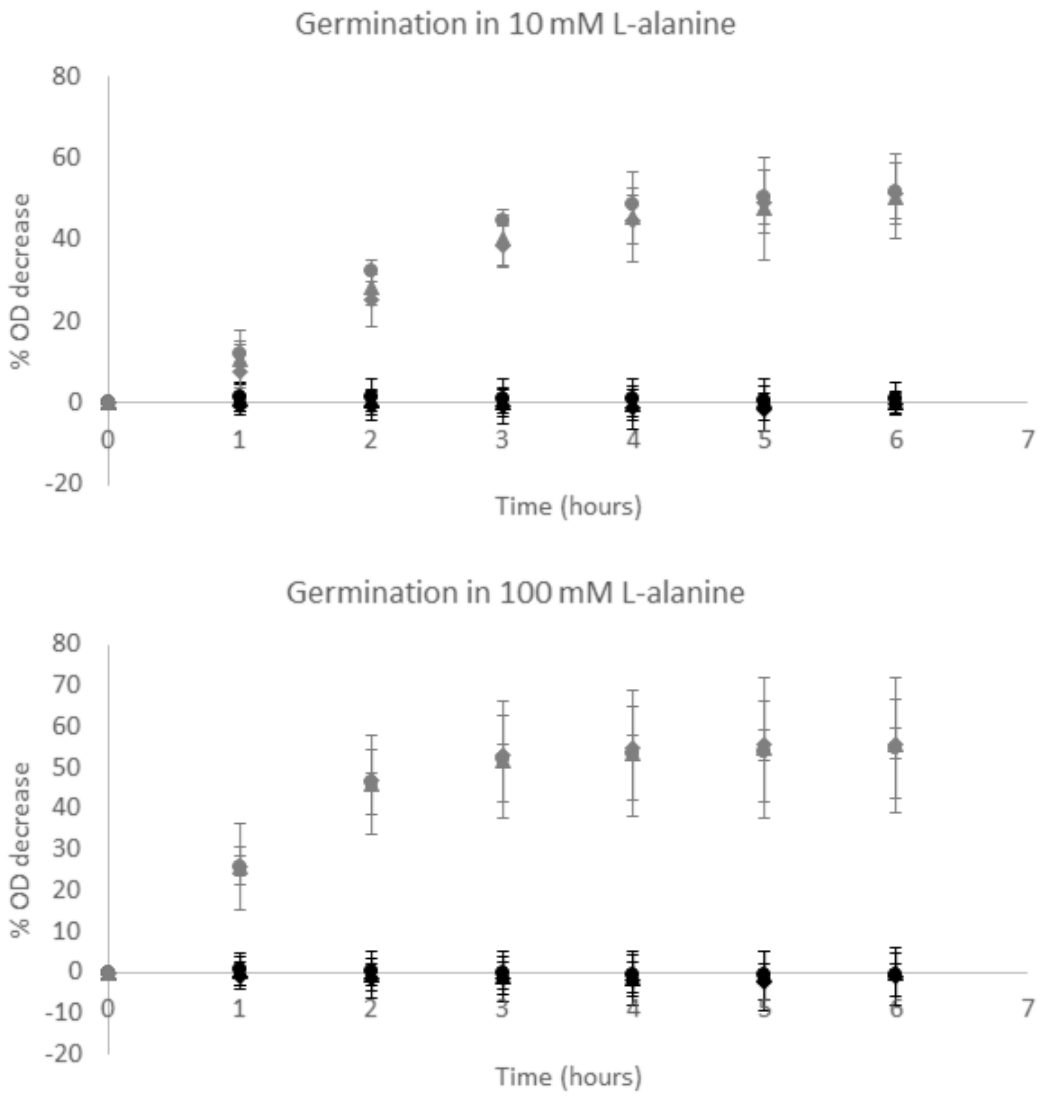

Germination in 10 mM L-alanine
% OD decrease
80
60
40
20
0
-20
0
1
2
3
4
5
6
7
Time (hours)
Germination in 100 mM L-alanine
% OD decrease
80
70
60
50
40
30
20
10
0
-10
-20
0
1
2
3
4
5
6
7
Time (hours)

Figure 6

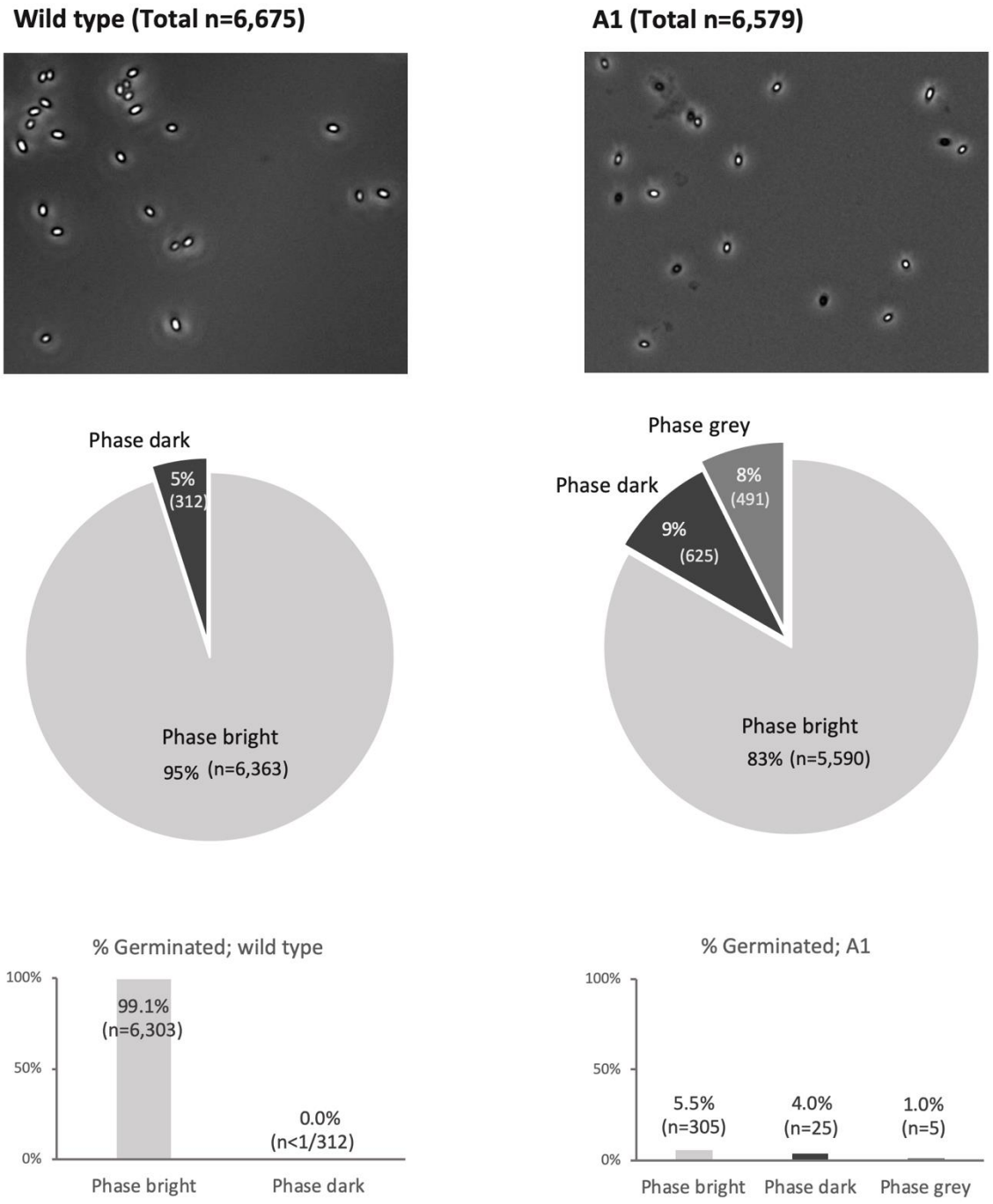
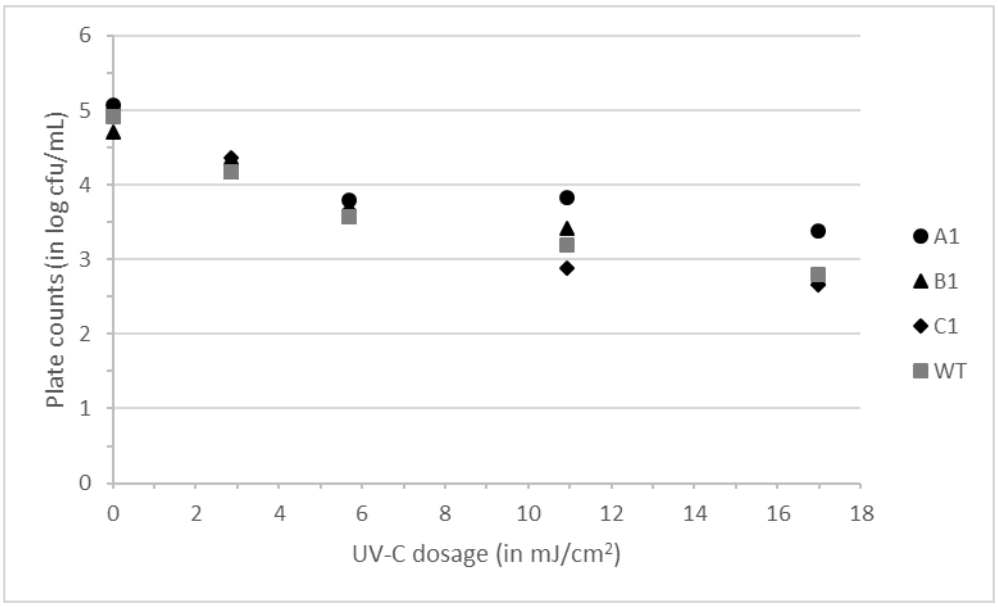

Wild type (Total n=6,675)
A1 (Total n=6,579)
Phase dark
5%
(312)
Phase bright
95% (n=6,363)
Phase grey
Phase dark
8%
(491)
9%
(625)
Phase bright
83% (n=5,590)
% Germinated; wild type
100%
50%
0%
99.1%
(n=6,303)
0.0%
(n<1/312)
Phase bright
Phase dark
% Germinated; A1
100%
50%
0%
5.5%
(n=305)
4.0%
(n=25)
1.0%
(n=5)
Phase bright
Phase dark
Phase grey

Figure 7



Highlights:

- *B. cereus* endospore UV-C resistance is a readily evolvable trait
- UV-C resistant mutant endospores display germination defects and heat sensitivity
- Endospore UV-C resistance is not transmitted to vegetative cell level