Food Microbiology 82 (2019) 36–45

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

# Mechanism of inactivation of *Bacillus subtilis* spores by high pressure $CO_2$ at high temperature

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ARTICLE INFO

*Keywords:*
*Bacillus subtilis*
Spores
High pressure $CO_2$
Inactivation
Mechanism

ABSTRACT

Spores of wild-type *Bacillus subtilis* and some isogenic mutant strains were treated by high pressure $CO_2$ (HPCD) at high temperature (HT) (HPCD + HT) at 20 MPa and 84-86 °C for 0–60 min, and centrifuged on a high density solution to obtain pelleted spores that retained CaDPA and light spores that lost CaDPA. All treated spores were analyzed for viability, and tested for germination, outgrowth, core protein damage, mutagenesis and inner membrane (IM) properties. The results showed that (i) with HPCD + HT treated spores, most pelleted spores and all light spores were dead; ii) a significant amount of dead HPCD + HT-treated spores that retained CaDPA germinated, but outgrowth was blocked; (iii) minimal mutants were generated in survivors of HPCD + HT treatment; (iv) the GFP fluorescence decrease in HPCD + HT-treated spores with high GFP levels was slower than spore inactivation; (v) the IM of HPCD + HT-treated spores that retained CaDPA lost its ability to retain CaDPA at 85 °C, and almost all of these spores' outgrowth in high salt was blocked; and (vi) HPCD + HT-treated spores that retained CaDPA germinated with L-valine or AGFK were almost all stained with propidium iodide. These results indicated that HPCD + HT inactivated *B. subtilis* spores by damaging spores' IM, thus blocking spore outgrowth after germination.

## 1. Introduction

Spores of bacteria of *Bacillus* and *Clostridium* species are extremely resistant to a variety of treatments including heat, desiccation, chemicals and radiation (Leggett et al., 2012; Nicholson et al., 2000; Setlow, 2006). As spores of some species can cause food spoilage or food-borne diseases (Brown, 2000; Logan, 2012; Wells-Bennik et al., 2016), inactivating spores during food processing is of great significance for the food industry. Traditionally, spores in food are inactivated by moist heat at 110–130 °C for 20–40 min, and sometimes treatment at 80–100 °C for 10 min is also sufficient to inactivate spores when the food is high acid or stored at cold conditions (Block, 2001; Earle, 1983). Although heat treatment is efficient at inactivating spores, it can also cause detrimental effects to heat sensitive foods. Thus, there is interest in developing treatments that can effectively inactivate spores while having minimal effects on food quality. One treatment that has attracted such interest is high pressure carbon dioxide ($CO_2$) (HPCD) at pressures < 50 Mega Pascals (MPa) and temperatures < 60 °C (Damar and Balaban, 2006; Ferrentino and Spilimbergo, 2011; Spilimbergo and Bertucco, 2003). HPCD treatment is very efficient at pasteurization, and can preserve more high-quality food attributes than traditional treatments (Bi et al., 2011; Ferrentino et al., 2009; Ferrentino and Spilimbergo, 2011). However, while HPCD can effectively inactivate vegetative forms of pathogenic and spoilage bacteria, yeasts, and molds (Garcia-Gonzalez et al., 2007; Perrut, 2012; Rao et al., 2016a; Zhang et al., 2006), generally higher temperatures (HT) (60-90 °C) are required for efficient bacterial spore inactivation by HPCD (Ballestra and Cuq, 1998; Rao et al., 2015; Watanabe et al., 2003). However, since temperatures of 60–90 °C only cause relatively mild damage to foods (Hwang and Huang, 2010; Rodney, 2004), HPCD combined with HT (HPCD + HT) could be a promising technique to both inactivate spores and maintain food quality.

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https://doi.org/10.1016/j.fm.2019.01.014
Received 9 April 2018; Received in revised form 25 January 2019; Accepted 25 January 2019
Available online 29 January 2019
0740-0020/ 

A previous study found that HPCD + HT treatment at 82-91 °C could effectively inactivate *Bacillus subtilis* spores (Rao et al., 2015). Further study indicated that the spores were probably inactivated by directly damaging spore structures such as the coat, cortex, inner membrane (IM) or core, rather than spores being first germinated and then inactivated (Rao et al., 2015, 2016b). However, which spore structure's or component's damage caused spore inactivation by HPCD + HT was not clear. Since generation of a growing cell from a spore - i.e. spore revival – requires spore germination and outgrowth, damage to any components or structures essential for these processes can lead to failure in spore revival. For *Bacillus* spores, germination and outgrowth take place as follows. 1) First, germinants permeate into spores and interact with and activate germinant receptors (GRs) in the IM. 2) Spores then become committed to germinate, such that germination proceeds even if germinants are removed or further GR-germinant interaction is blocked (Setlow, 2013; Setlow et al., 2017; Yi and Setlow, 2010). 3) The germination signal is then transduced in some fashion leading to Stage I of germination (Setlow, 2013, 2014; Setlow et al., 2017), which begins by release of spores' monovalent cations and then release of spores' large depot (~25% of spore core dry wt) of pyridine-2, 6-dicarboxylic acid (dipicolinic acid (DPA)); DPA is present only in the spore core, and as a 1:1 chelate with divalent cations, primarily $Ca^{2+}$ (CaDPA). CaDPA is released via specific channels in spores IM composed largely or perhaps completely of multiple SpoVA proteins. Replacement of the released CaDPA by water raises spore core water content somewhat, and completes Stage I of germination. 4) CaDPA release in Stage I then initiates Stage II by activating cortex-lytic enzymes (CLEs) that initiate the degradation of spores' large peptidoglycan (PG) cortex that is likely important in maintaining the low water content in the dormant spore core. Degrading cortex PG then allows core expansion and water uptake to levels found in growing cells and completion of germination. At this point the outgrowth of the germinated spore begins as enzymes become active in the core leading to initiation of metabolism and macromolecular synthesis that converts the germinated spore into a growing cell (Paidhungat and Setlow et al., 2000). Another important event in outgrowth is the complete degradation of a group of α/β-type small, acid-soluble proteins (SASP) that saturate dormant spore DNA and protect it from many types of damage (Setlow, 2006).

Obviously, severe damage to a number of spore structures or components could lead to spore death, and five different mechanisms of spore killing have been identified (Doona et al., 2015; Setlow et al., 2014; Setlow, 2006). These killing mechanisms are: 1) rupture of spores' IM by high concentrations of strong mineral acids (Setlow et al., 2002; Setlow, 2006); 2) damage to spores' IM by oxidizing agents such as chlorine dioxide, hypochlorite or iodine as well as a combination of HPCD and peracetic acid, such that the IM ruptures after the treated spores germinate (Cortezzo et al., 2004; Leggett et al., 2012; Li et al., 2017; Setlow et al., 2016; Setlow, 2006); 3) inactivation of essential germination components by strong alkali or a high concentration of $CaCl_2$ at 80 °C, although spores treated in this way can be recovered by artificial germination (Rao et al., 2016c; Setlow et al., 2002); 4) damage to some core protein essential for spore outgrowth by wet heat or hydrogen peroxide (Coleman et al., 2007, 2010; Melly et al., 2002; Wang et al., 2011); and 5) DNA damage leading to mutagenesis by agents such as UV or λ radiation, dry heat, or genotoxic chemicals such as nitrous acid and formaldehyde (Setlow, 2006). With so many different mechanisms for spore inactivation, obviously a major question about spore killing by HPCD + HT is how does this agent kills spores?

Previous results showed that loss of spores' CaDPA depot was slower than loss of spore viability during HPCD treatment plus either HT or peracetic acid (Rao et al., 2016b; Setlow et al., 2016). It has also been reported that CaDPA release from an individual spore is an all-or-nothing phenomenon at least during moist-heat killing (Coleman et al., 2007, 2010; Zhang et al., 2010, 2011). These findings suggest that analysis of the properties of HPCD + HT-treated spores that retain CaDPA and are killed to various levels could give valuable information on the mechanism(s) of HPCD + HT killing. Consequently, in this work, we centrifuged HPCD + HT-treated spores on a very high-density solution to isolate spores that did and did not retain CaDPA (Coleman et al., 2007, 2010). The properties of both types of recovered spores were then measured by examining a number of spore properties such as: 1) IM permeability by staining of nucleic acid in the spore core (Setlow et al., 2016); 2) spore germination triggered by GR-dependent and GR-independent germinants (Rao et al., 2016c; Setlow et al., 2016); 3) core protein damage by monitoring the fluorescence intensity of green fluorescent protein (GFP) in spores that overexpress this protein in the developing spore core (Coleman et al., 2007); and 4) testing for DNA damage by examining survivors of HPCD + HT treatment for mutations (Huesca Espitia et al., 2016; Li et al., 2017; Setlow et al., 2016).

## 2. Materials and Methods

### 2.1. Bacillus subtilis strains and spore preparation

All *B. subtilis* strains used are isogenic with a laboratory 168 strain, and are: (i) PS533 (wild-type), carrying plasmid pUB110 encoding resistance to kanamycin ($Km^r$; 10 μg/ml) (Setlow and Setlow, 1996); (ii) PS578 ($\alpha^-\beta^-$) (Setlow and Setlow, 1996), identical to PS533 but with deletions in the *sspA* and *sspB* genes encoding the major DNA protective α/β-type SASP that comprise ~ 85% of spores' total α/β-type SASP (Setlow and Setlow, 1996; Setlow, 2006); (iii) PS2318 (*recA*) (Young and Setlow, 2003), lacking the *recA* gene essential for repair of many types of DNA damage, and carrying a gene encoding resistance to erythromycin ($Em^r$; 1 μg/ml) (Setlow, 2006); (iv) PS3328 (*cotE*) (Paidhungat et al., 2001), lacking the *cotE* gene resulting in a very defective spore coat, and carrying a gene encoding resistance to tetracycline ($Tc^r$; 10 μg/ml); and (v) PS3518 (hiGFP) (Cowan et al., 2003; Webb et al., 1995), carrying the *gfp* gene encoding GFP under the control of the strong forespore-specific promoter of the *B. subtilis sspE* gene, so that spores of this strain accumulate very high GFP levels in the spore core. The strain is also $Km^r$. Spores of all strains were prepared at 37 °C on 2 × Schaeffer's-glucose agar plates without antibiotics as described previously (Nicholson and Setlow, 1990; Paidhungat et al., 2000). All spores used were free (> 98%) of germinated spores, growing or sporulating cells, or cell debris as observed by phase contrast microscopy, and were stored in water at 4 °C protected from light.

### 2.2. HPCD + HT treatment

Spores were treated by HPCD + HT at 20 MPa and 84-86 °C for 0–60 min with a batch HPCD system (Liao et al., 2007). For each experiment, 20 ml of spores at an optical density at 600 nm ($OD_{600}$) of 5 (~$1.5 \times 10^9$ CFU/ml) suspended in sterile distilled water in 50 ml sterile glass tube were placed into the pressure vessel when the vessel reached the desired experimental temperature, then the vessel was pressurized to 20 MPa by the plunger pump within 2.5 min. After holding for the required treatment time, the vessel was depressurized within 2.5 min, and the spores were removed from the vessel, cooled on ice and analyzed immediately. Spore treatment by HT at 0.1 MPa and 84-86 °C and without $CO_2$ was in a water bath, again with 20 ml of spores at $OD_{600}$ of 5 in a 50 ml sterile glass tube. After treatments, spores were removed from the water bath and analyzed immediately after cooling.

### 2.3. Enumeration of surviving spores

Untreated, HT-treated and HPCD + HT-treated spores at $OD_{600}$ of 1 were serially diluting 1/10 in cold sterile distilled water, aliquots (10 μl) were spotted on LB medium plates containing appropriate antibiotics (Paidhungat et al., 2000). The plates were incubated at 37 °C

for 18–36 h until there was no further increase in numbers of colonies, and then colonies were counted.

### 2.4. Fractionation of HPCD + HT treated spores

HPCD + HT treated spores were fractionated by centrifugation on a high density solution as described previously (Coleman et al., 2007, 2010). Spores (100 µl) at $OD_{600}$ of 100–200 suspended in 20% Histodenz were layered on 50% Histodenz in a 1.5 ml microcentrifuge tube, and centrifuged at 25 °C for 10 min at 12, 000 × *g* in a 5418R ultracentrifuge (Eppendorf, Germany). After centrifugation, the spores that retained CaDPA (see Results) pelleted and were termed pelleted fraction, and spores that had lost CaDPA (see Results) banded close to the top of tube and were termed the upper band fraction. The spores that pelleted and banded were removed, washed five times with sterile distilled water to remove Histodenz, suspended in cold sterile distilled water at $OD_{600}$ of 2 and stored at 4 °C prior to further analysis.

### 2.5. Measurement of spore germination and outgrowth

GR-dependent spore germination was triggered by L-valine or AGFK (a mixture of L-asparagine, D-glucose, D-fructose, and KCl). Prior to germination, spores at an $OD_{600}$ of 10 were heat activated at 75 °C for 30 min (L-valine germination) or 2 h (AGFK germination), and cooled on ice for at least 15 min (Luu et al., 2015; Setlow et al., 2016; Yi and Setlow, 2010). Spores at an $OD_{600}$ of 0.5 were germinated at 37 °C in a 96-well plate in 200 µl of 25 mmol/l K-Hepes buffer (pH 7.4) and 10 mmol/l L-valine or AGFK (10 mmol/l in each AGFK component) with 50 µmol/l $TbCl_3$. Spore germination was monitored by measuring Tb-DPA fluorescence in a Spark 10M multi-well fluorescence plate reader (Tecan, Switzerland) as described previously (Yi and Setlow, 2010). The degree of germination was also examined at the end of incubations by phase contrast microscopy (PCM) to confirm that the levels of germination determined by monitoring DPA release were consistent with levels determined by PCM. In contrast to GR-dependent germination, Germination by dodecylamine does not require heat activation (Setlow, 2013), and was conducted at 50 °C with spores at an $OD_{600}$ of 0.5 in 4 ml of 25 mmol/l K-Hepes buffer and 1 mmol/l dodecylamine. At various times, 150 µl of the germination incubations were added to 50 µl of 200 µmol/l $TbCl_3$ and 100 mmol/l K-Hepes buffer (pH 7.4), and the Tb-DPA fluorescence was measured as described above.

To monitor both spore germination and outgrowth, spores at $OD_{600}$ of 0.8 were incubated at 37 °C in LB medium with 10 mmol/l L-valine (Paidhungat and Setlow, 2000). At various times, spores were examined by PCM to determine the % of dormant spores (phase bright), germinated spores (phase dark), outgrowing spores (beginning to elongate) and growing cells.

All the data for spore germination and outgrowth is from one experiment, but all experiments were repeated at least in triplicate with similar results.

### 2.6. Measurement of spore coat damage

Spore coat damage was examined by testing spore resistance to hypochlorite as described previously, since an intact spore coat is essential for full hypochlorite resistance (Ghosh et al., 2008; Klobutcher et al., 2006; Young and Setlow, 2003). Spores at an $OD_{600}$ of 1 were treated at 25 °C for 1 h with sodium hypochlorite with 0.15% available chlorine in 50 mmol/l $KPO_4$ buffer (pH 7.0). At various times, spores were diluted 1/10 in 1% sodium thiosulfate, incubated at 25 °C for 10 min and diluted further in sterile distilled water. Surviving spores were enumerated as described above.

### 2.7. Measurement of DNA damage

DNA damage following HPCD + HT treatment was measured by determining the levels of mutations in survivors of the HPCD + HT treatment of wild-type spores to either asporogeny (*spo*) or auxotrophy (*aux*). Assessment of mutagenesis in survivors of HPCD + HT treatment was as described previously (Huesca Espitia et al., 2016; Li et al., 2017; Setlow et al., 2016). 200 µl of appropriate dilutions of wild-type spores of untreated or treated by HPCD + HT or UV irradiation at 254 nm (Huesca Espitia et al., 2016; Setlow et al., 2014) (> 99% inactivation, see Results) were spread on LB medium plates with 10 µg/ml kanamycin. After overnight incubation at 37 °C, ~400 individual colonies were transferred to sporulation agar plates and Spizizen's minimal medium plates without Casamino acids (Spizizen, 1958). The plates were incubated for 2–3 days at 37 °C, and were examined for asporogenous (*spo*) and auxotrophic (*aux*) mutations as described previously (Fairhead et al., 1993).

The resistance of wild-type, $\alpha^-\beta^-$ and *recA* spores to HPCD + HT and to HT treatment alone was also tested, since in particular $\alpha^-\beta^-$ spores are expected to be more sensitive to HT treatment than either wild-type or *recA* spores, while recA spores would be more sensitive than wild-type spores to HPCD + HT if this treatment kills spores by DNA damage (Li et al., 2017; Setlow et al., 2016; Setlow, 2006).

### 2.8. Measurement of spore core protein damage

Spore core protein damage was examined by monitoring fluorescence decay of GFP in the spore core using spores of strain PS3518 (hiGFP) during HPCD + HT treatment (Coleman et al., 2007). Untreated and HPCD + HT treated spores were examined by an Eclipse Ti inverted fluorescence microscope (Nikon, Japan) equipped with a × 100 objective lens using FITC filter with excitation at 465–495 nm and emission at 512–558 nm. The spores were also directly tested for fluorescence intensity using an Accuri C6 flow cytometry (BD Accuri Cytometer Inc., USA) with the green fluorescence (FL1) detector at 530 nm ± 15 nm. Spores were analyzed at a nominal flow rate of 14 µl/min with a stream core diameter of 10 µm. All samples were evaluated after 30,000 events were recorded.

### 2.9. Measurement of IM damage

To test the spore IM's ability to retain CaDPA, untreated and HPCD + HT-treated spores, 200 µl at $OD_{600}$ of 0.5, were incubated at 85 °C for 45 min, cooled on ice, centrifuged at 25 °C for 10 min at 10, 000 × *g*, 150 µl of the supernatant fluid added to 50 µl of 200 µmol/l $TbCl_3$ and 100 mmol/l K-Hepes buffer (pH 7.4), and CaDPA release was quantified from the Tb-DPA fluorescence as described above (Yi et al., 2011; Yi and Setlow, 2010).

To test spores' ability to outgrow in a high salt environment, likely a reflection of the state of spores' IM that becomes the outgrowing spore's plasma membrane, treated and HPCD + HT-treated spores were spotted on LB plates with 0.15 mol NaCl/l, 1 mol NaCl/l or 1 mol NaCl plus 50 mmol/l D-glucose and the viability on these plates was determined as described above (Cortezzo et al., 2004).

To examine the IM's permeability after germination, spores were germinated with L-valine or AGFK for 140 min as described above, stained with 15 µmol/l propidium iodide (PI), and photographed with an Eclipse Ti inverted fluorescence microscope (Nikon, Japan) equipped with a × 100 objective lens using TRITC filter with excitation at 527–553 nm and emission at 577–633 nm. The distribution of the fluorescence intensity in individual PI-stained germinated spores was determined using an Accuri C6 Flow Cytometer (BD Accuri Cytometer Inc., USA) equipped with a 488 nm, 50 mW laser. Fluorescence from PI staining was quantified with the FL2 detector at 585 nm ± 15 nm, and the other parameters were the same as assessment for fluorescence intensity for GFP as described above. Untreated dormant spores as well as spores in the upper band and pellet fractions from HPCD + HT-treated spores were also stained with PI and analyzed as described above.

### 2.10. Data analysis

Flow cytometry data were analyzed using the FlowJo version 7.6.1 software (FlowJo, OR, USA). Analysis of variance was carried out by PASW statistic 18 (SPSS, USA) to determine significance at 0.05 level. All experiments were carried out at least in triplicate.

## 3. Results and discussion

### 3.1. Spores treated by HPCD + HT exist in states of dormant, dead and retaining CaDPA, and dead and without CaDPA

Spores of *Bacillus* species have a relatively high wet density, mostly because of the high CaDPA and low water content in the spore core (Popham et al., 1996; Setlow et al., 2001). Consequently, CaDPA release and its replacement by water leads to a significant decrease in spore wet density, and spores that retain CaDPA can be easily be separated from spores that have lost CaDPA by centrifugal fractionation on solutions of high density (Coleman et al., 2007, 2010). As noticed previously, *B. subtilis* spore inactivation during HPCD + HT treatment was always faster than was CaDPA release (Rao et al., 2015, 2016b). Therefore, we speculated that while many HPCD + HT-treated spores are dead, most retain their CaDPA. To examine this hypothesis, wild-type and hiGFP spores treated by HPCD + HT for 30 min were tested for viability and then fractionated by centrifugation on a solution of high density. As predicted, for both wild-type and hiGFP spores, most of the HPCD + HT-treated spores that pelleted in the high density solution were dead but retained all CaDPA (Table 1). In contrast, the lower density HPCD + HT-treated spores in the upper band were all dead and retained no CaDPA (Table 1). Therefore, spores existed in three different states including dormant, dead and retaining CaDPA and dead and without CaDPA with different proportions during HPCD + HT treatment (Table 1). This is also the case for inactivation spores by moist heat (Coleman et al., 2007, 2010). The HPCD + HT-treated spores that had lost CaDPA could be largely stained by PI (Table 1), indicating the rupture of their IM. In contrast, HPCD + HT-treated spores that retained CaDPA could not be stained by PI (Table 1), indicating that the IM of these spores was not or only mildly damaged. Presumably, the dead spores retaining CaDPA must have been inactivated more recently than the dead spores that had lost CaDPA. Therefore, determining why the dead spores that retained CaDPA spores were dead could be of great importance for understanding the mechanism of inactivation of spores by HPCD + HT. Importantly, simply the existence of the dead spores that retained CaDPA is strong evidence that HPCD + HT treatment did not inactivate spores by rupturing the dormant spore IM, as this would have led to rapid CaDPA release.

### 3.2. HPCD + HT-treated spores that retained CaDPA could be partially germinated but were blocked in outgrowth

The reason that HPCD + HT-treated spores that retained CaDPA almost all appeared dead could be because the germination and/or outgrowth of these treated spores was blocked. Thus, the germination and outgrowth of HPCD + HT inactivated spores that retained CaDPA were studied. The germinants used were the GR-dependent germinants L-valine and AGFK that trigger germination via the GerA or GerB plus GerK GRs, respectively, and dodecylamine which directly opens the SpoVA protein channel for CaDPA (Setlow et al., 2017). With L-valine and AGFK, largely dead HPCD + HT-treated spores that retained CaDPA exhibited some germination, but this was less than with untreated spores (Fig. 1A and B). Specifically, the percent germination of these HPCD + HT-treated spores (≤0.2% viability) after 140 min incubation with L-valine or AGFK was ~20 and 37%, respectively, while ≥ 98% of untreated spores germinated with these two germinants (data not shown). Notably, the percent germination of the HPCD + HT-treated spores with the two GR-dependent germinants was much greater than these spores' viability, indicating that a significant percentage of the dead spores that retained CaDPA could germinate relatively normally. Therefore, the death of these HPCD + HT-treated spores that retained CaDPA was not due to damage to the GerA, GerB, GerK GRs, which might block spore germination. In contrast to the slower germination of HPCD + HT-treated spores with GR-dependent germinants compared to that of untreated spores, these treated spores' germination with the GR-independent germinant dodecylamine was faster than that of the untreated spores, and ≥90% of both types of spores germinated in ~60 min (Fig. 1C), indicating that the HPCD + HT-treated spores that retained CaDPA germinated quite well with dodecylamine. This at least suggested that the SpoVA proteins of most of these treated spores were not damaged, but what caused the faster germination triggered by dodecylamine was not clear. It has been previously reported that *B. subtilis* spores with coat defects exhibit more rapid germination with dodecylamine than wild-type spores (Knudsen et al., 2016). Thus, we speculated that the high rate of dodecylamine triggered germination might be due to spore coat damage. Previous studies indicated that spores with coat defects are very sensitive to hypochlorite treatment (Ghosh et al., 2008; Klobutcher et al., 2006; Young and Setlow, 2003). Thus, we examined the hypochlorite killing of HPCD + HT-treated wild-type spores that retained CaDPA (Fig. 2). Notably, compared with untreated spores, the HPCD + HT-treated spores that retained CaDPA were more sensitive to hypochlorite, although exhibited lower hypochlorite sensitivity than *cotE* spores. The

**Table 1**
Viability and CaDPA content of HPCD + HT-treated spores.[a]

| Strains | Treatments | Viability (%) | CaDPA content (%) | PI staining (%) |
|---|---|---|---|---|
| PS533 (wild-type) | Untreated | 100a | 100a | 0 |
| | [1]HPCD + HT, total | 0.03 ± 0.01b | 9.1 ± 2.3c | 90.5 |
| | [2]HPCD + HT, upper band | 0.0004 ± 0.0001d | 1.2 ± 0.9e | 96.4 |
| | [3]HPCD + HT, pellet | 0.17 ± 0.12f | 97.9 ± 1.9a | 0.3 |
| PS3518 (hiGFP) | Untreated | 100a | 100a | – |
| | [1]HPCD + HT, total | 0.05 ± 0.03b | 10.7 ± 1.5c | – |
| | [2]HPCD + HT, upper band | 0.0003 ± 0.0002d | 1.4 ± 1.1e | – |
| | [3]HPCD + HT, pellet | 0.20 ± 0.11f | 96.6 ± 3.1a | – |

[1]HPCD + HT treatment was carried out at 20 MPa and 84-86 °C for 30 min and the total spores after treatment were analyzed; HPCD + HT-treated spores that
[2]banded close to the top (upper band)
[3]pelleted on the bottom upon centrifugation on a high density solution as described in Materials and Methods were analyzed. Different letters indicate significant differences in the viability and CaDPA content ($p < 0.05$).
'-' indicates not tested.
[a] Values for viability and CaDPA content for untreated spores of each strain were set at 100%, and all analyses were carried out as described in Materials and Methods.

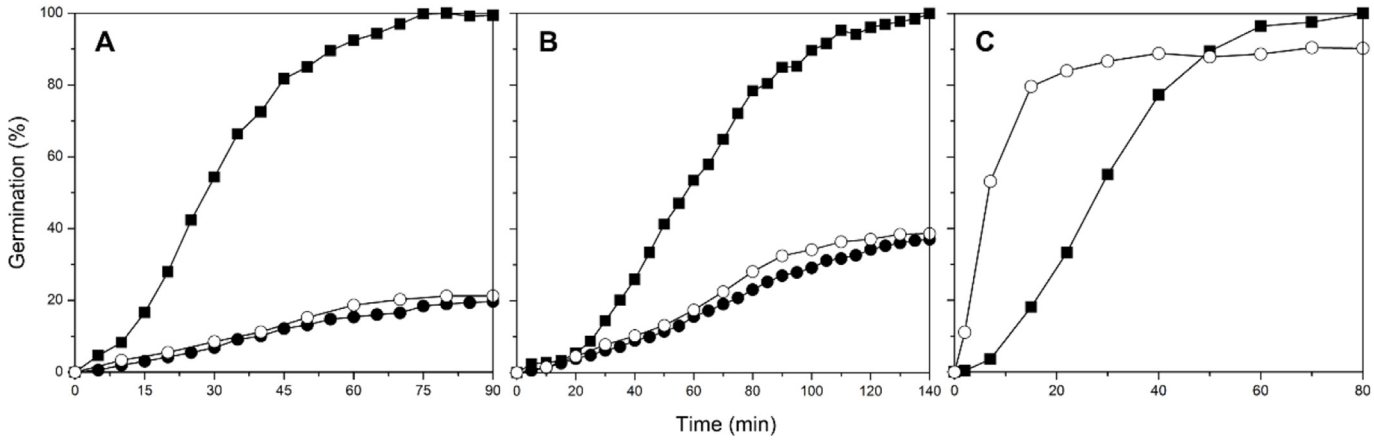


**Fig. 1.** Germination of untreated and HPCD + HT-treated PS533 (wild-type) spores. The symbols used are: (■) untreated spores germinated with $TbCl_3$, and treated spores that retained CaDPA from Table 1 germinated (●) with or (○) without $TbCl_3$. The germinants used were L-valine (A), AGFK (B), and dodecylamine (C); germination experiments were carried out as described in Materials and Methods. All the data for spore germination is from one experiment, but all experiments were repeated at least in triplicate with similar results.

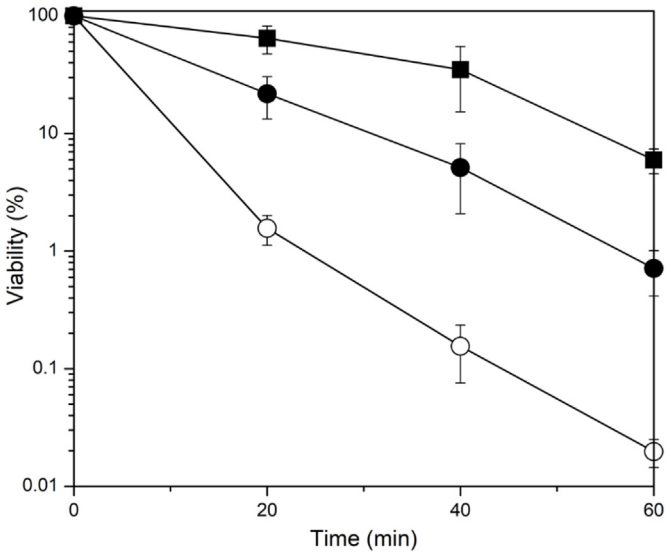


**Fig. 2.** Inactivation of untreated or HPCD + HT-treated spores by sodium hypochlorite. Symbols used are: (■) untreated PS533 (wild-type) spores; (●) HPCD + HT-treated PS533 spores that retained CaDPA and described in Table 1; and (○) untreated PS3328 (*cotE*) spores. Sodium hypochlorite treatment and assessment of spore viability were carried out as described in Materials and Methods. Values shown are from triplicate determinations at each time point, and standard deviations for these measurements are shown. Similar results were obtained in three independent experiments.

latter results indicated that HPCD + HT treatment caused some damage to spores' outer layers, in particular the coat, and may be the reason for increased rate of dodecylamine germination of HPCD + HT treated spores that retained CaDPA (Knudsen et al., 2016). However, $Tb^{3+}$ had no effect on the L-valine or AGFK triggered germination of the HPCD + HT-treated spores that retained CaDPA (Fig. 1A and B). Thus, the coat damage of these HPCD + HT-treated spores was not severe enough for $Tb^{3+}$ to gain access into more spore inner layers and inhibit germination as reported previously (Knudsen et al., 2016; Yi et al., 2011).

Since the HPCD + HT inactivated spores that retained CaDPA could germinate, the death of these spores was probably due to abolishing outgrowth after germination. Therefore, we examined the germination and outgrowth of HPCD + HT-treated spores that retained CaDPA by PCM of spores incubated in liquid LB growth medium plus L-valine (Fig. 3). As shown in Fig. 3 and Table S1, the untreated spores germinated normally and outgrew to vegetative cells, as expected. For the HPCD + HT-treated spores that retained CaDPA, many could germinate as expected and became phase dark, indicating that the cortex was degraded and the CLEs functioned well (Setlow et al., 2017). Thus, the death of these spores was not due to damage to the CLEs. However, the outgrowth for these HPCD + HT-treated spores after germination was obviously blocked, suggesting that spore components or structures involved in spore outgrowth including DNA, core protein, or the IM were damaged (Doona et al., 2015; Setlow et al., 2016; Setlow, 2006; Wang et al., 2011).

### 3.3. The outgrowth defect of HPCD + HT-treated spores that retained CaDPA was not due to DNA damage

Previous studies reported that 5–10% of the survivors of *B. subtilis* spores treated with DNA damaging agents such as UV radiation or genotoxic chemicals such as nitrous acid contained either *spo* or *aux* mutations (Setlow et al., 2014; Setlow, 2006). Since it was possible that HPCD + HT treatment could damage spore DNA, and inactivate spores by blocking spore outgrowth, we examined a large number of survivors from HPCD + HT-treated spores for these mutations (Table 2). Notably, less than 0.25% of survivors from total HPCD + HT-treated spores had mutations, and no mutants were found in survivors from treated spores that retained CaDPA, while ~7% mutants were found in UV treated spores (Table 2). These results strongly suggested that HPCD + HT did not inactivate spores by damaging DNA. Moreover, when spores of isogenic wild-type, $\alpha^-\beta^-$, and *recA B. subtilis* strains were treated by HPCD + HT (Fig. 4A and B) or HT alone (Fig. 4B), $\alpha^-\beta^-$ spores were more rapidly inactivated and to a higher level than wild-type or *recA* spores (Fig. 4A). When HPCD + HT treatment was carried out for 30 min, the inactivation of the wild-type, $\alpha^-\beta^-$, and *recA* spores was 3.4 log, 5.5 log and 3.0 log, respectively (Fig. 4B). For HT treatment for 30 min, the inactivation of the wild-type, $\alpha^-\beta^-$, and *recA* spores was 0.15 log, 3.41 log, and 0.72 log, respectively (Fig. 4B). These data indicated that *recA* spores had similar resistance to HPCD + HT (Fig. 4A and B) or HT (Fig. 4B) as wild-type spores, while $\alpha^-\beta^-$ spores were more sensitive to both HPCD + HT (Fig. 4A and B) or HT (Fig. 4B) than wild-type spores. Overall, the results in Fig. 4 suggested that (i) lack of the DNA repair protein RecA had no major effect on spore resistance to HT or HPCD + HT; but that (ii) the absence of the majority of DNA protective α/β-type SASP decreased spore resistance to HPCD + HT, almost certainly because of these spores much lower resistance to wet heat alone (Fig. 4B), as has been found previously (Setlow, 2006). All

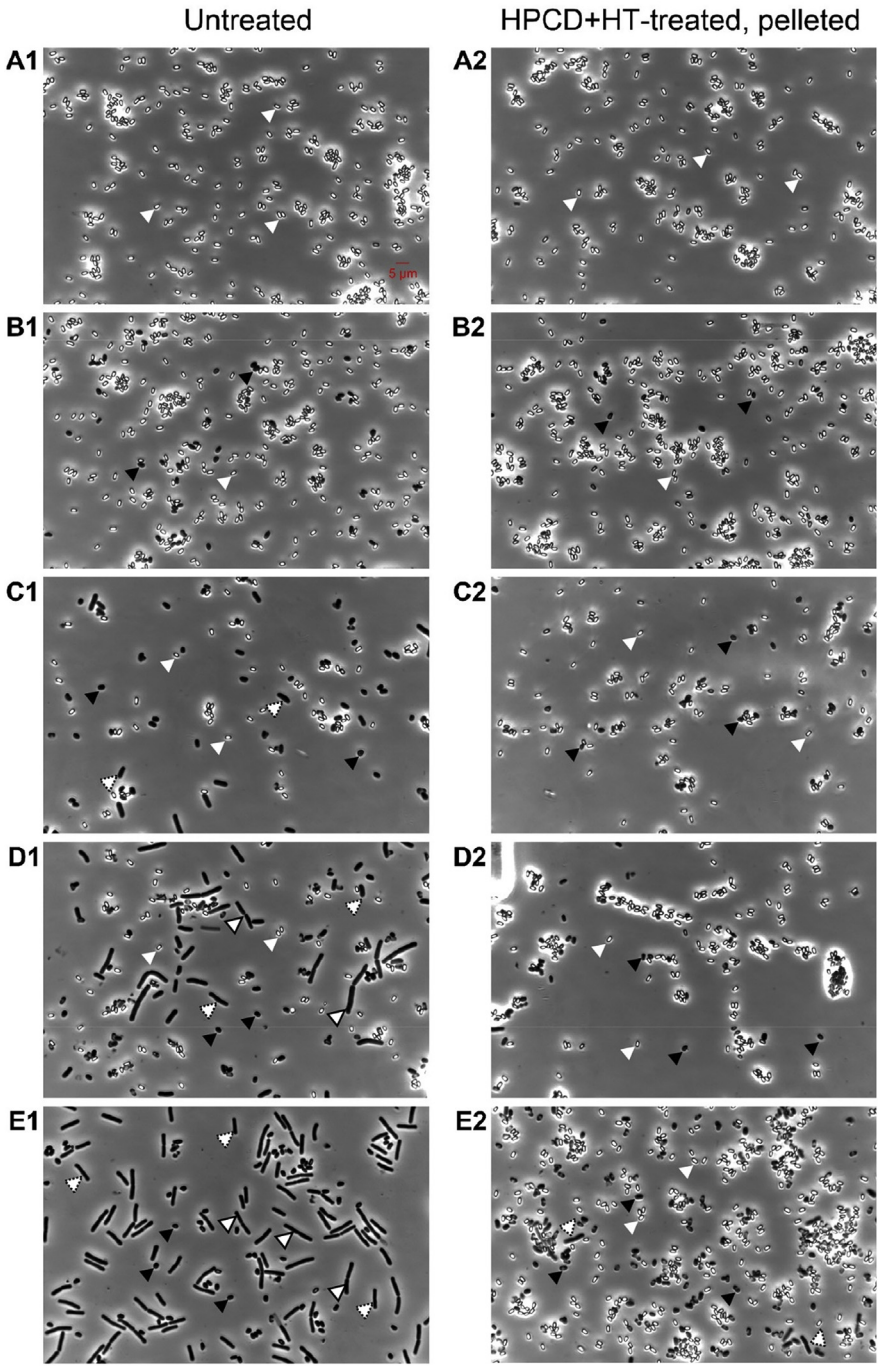


**Fig. 3.** Phase contrast photomicrographs of untreated (A1-E1) or HPCD + HT-treated (30 min) (A2-E2) PS533 (wild-type) spores during germination and outgrowth for 0 min (A1, A2), 60 min (B1, B2), 120 min (C1, C2), 180 min (D1, D2), 240 min (E1, E2). The HPCD + HT-treated spores are those that retained CaDPA described in Table 1. Black arrows indicate dormant spores, green arrows indicate germinated spores, blue arrows indicate elongating germinated spores, and red arrows indicate vegetative cells derived from outgrown spores. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
Levels of mutations generated by HPCD + HT or UV treatment of wild-type spores.

| Treatment | Inactivation (%) | Number of mutants | | |
|---|---|---|---|---|
| | | *aux* | *spo* | *aux spo* |
| Untreated | 0 | 0 | 0 | 0 |
| [1]HPCD + HT, total | 99.97 | 0 | 1 | 0 |
| [2]HPCD + HT, pellet | 99.83 | 0 | 0 | 0 |
| UV irradiation | 99.28 | 8 | 19 | 2 |

The HPCD + HT-treated [1]total spores and those that [2]pelleted and retained CaDPA were those used in Table 1. ~ 400 individual colonies were screened for *aux* or *spo* mutants in each experiment as described in Materials and Methods. All data in this table is from one experiment, but all experiments were repeated at least in triplicate with similar results.

these results suggested that HPCD + HT treatment did not inactivate wild-type spores by DNA damage.

### *3.4. The outgrowth defect of HPCD + HT-treated spores that retained CaDPA was likely not due to core protein damage*

Since HPCD + HT treatment could well denature some spore core protein(s) essential for spore outgrowth, we used spores containing a large amount of GFP in the spore core (Coleman et al., 2007), and examined spore GFP fluorescence by fluorescence microscopy and flow cytometry. In this experiment, spore viability and GFP fluorescence intensity (FI) were both set at 100% for untreated spores (Fig. 5, Table S2). For 30 min HT-treated spores (Fig. 5), spore viability decreased only minimally and FI not at all (Table S2), indicating that HT treatment had no major deleterious effect on spore core GFP structure.

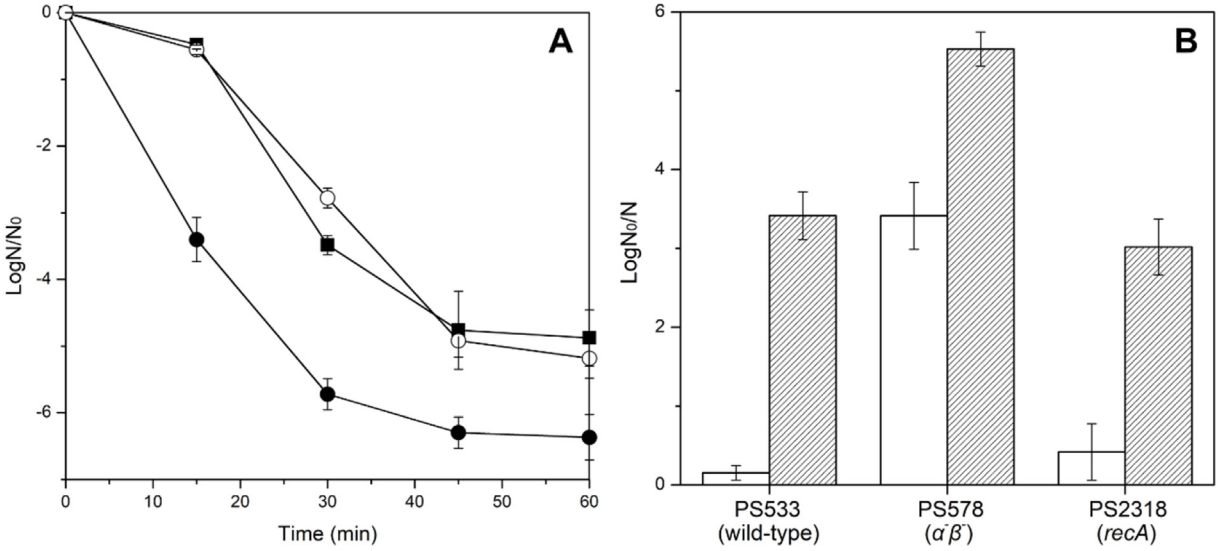


**Fig. 4.** Inactivation of spores of various *B. subtilis* strains by HPCD + HT (A, B) or HT alone (B). (A) Spores were treated with HPCD + HT and spore viability was determined as described in Materials and Methods; symbols used are: (■) PS533 (wild-type), (●) PS578 ($\alpha^-\beta^-$) and (○) PS2318 (*recA*). (B) Spores were treated with HPCD + HT or HT for 30 min and spore viability was determined as described in Materials and Methods; the bars used are: HPCD + HT (▨); and HT (□). $N_0$ and N are the survivors of spores treated at times zero and *t*, respectively. Values shown are from triplicate determinations, and standard deviations for these measurements are shown. Similar results were obtained in three independent experiments.

However, with spores treated with HPCD + HT for 30 min (Fig. 5; Table S2), the loss in spore viability was much faster than was loss of GFP fluorescence. This was also the case when only 30 min HPCD + HT-treated spores that retained CaDPA were examined (Fig. 5; Table S2). However, the 30 min HPCD + HT-treated spores that had lost all CaDPA (upper band spores) had lost almost all GFP fluorescence. The slower decrease of GFP fluorescence than spore inactivation during HPCD + HT treatment (Fig. 5, Table S2) indicates that the damage to GFP occurred after spore inactivation. As GFP and its fluorescence is generally stable at pH 7.5, but becomes very sensitive to HT treatment at 70 °C when the pH is lowered to 6.5 (Alkaabi et al., 2005), GFP would become very sensitive when treated by HPCD + HT at 20 MPa and 84-86 °C, which could generate pH as low as 3 (Meyssami et al., 1992; Spilimbergo et al., 2005). Thus, damage to other core proteins might occur even slower than to GFP during HPCD + HT treatment. Therefore, the core protein damage seen in HPCD + HT-treated spores probably occurred after spore inactivation, suggesting that the blocking of outgrowth of HPCD + HT-treated spores that retained CaDPA was not likely due to the core protein damage. However, at this time we cannot rule out the possibility that some essential spore core protein is inactivated in parallel with spore killing by HPCD + HT treatment.

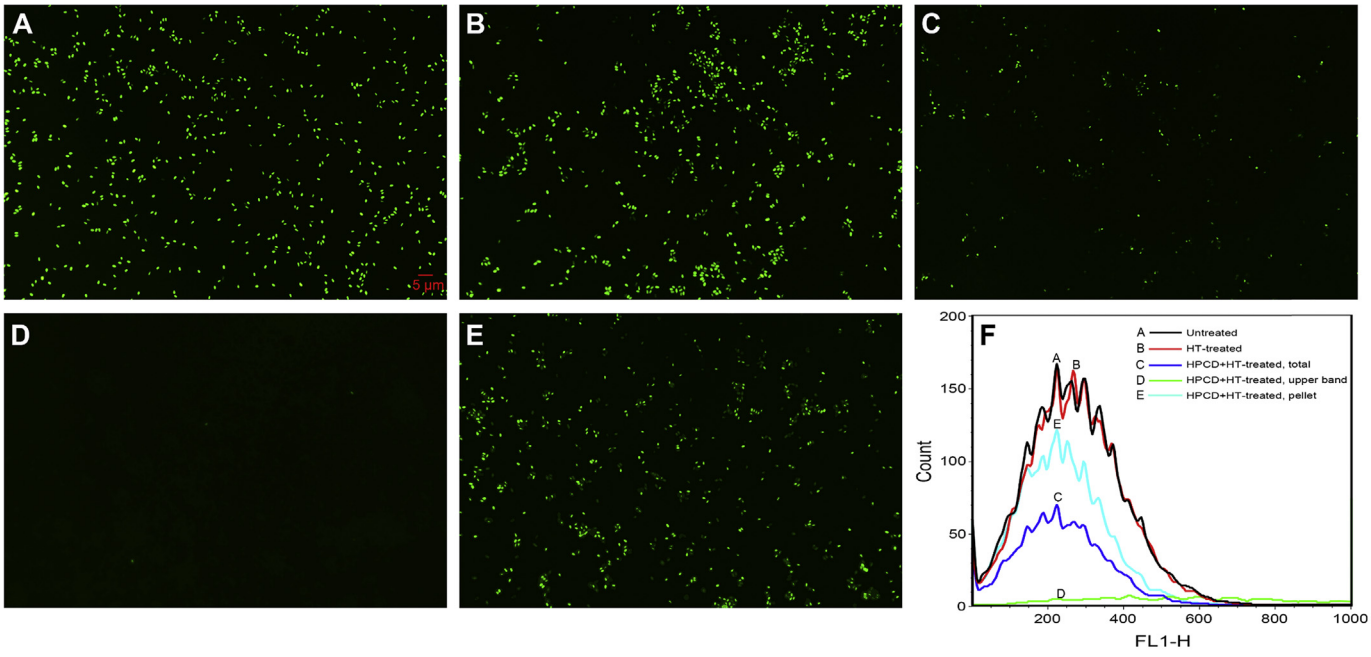


**Fig. 5.** Fluorescence photomicrographs (A–E) and flow cytometry histograms (F) of HPCD + HT- or HT-treated PS3518 (hiGFP) spores. Spores tested were (A) untreated; (B) HT-treated; (C) HPCD + HT-treated, total; (D) HPCD + HT-treated, upper band; (E) HPCD + HT-treated, pellet. Spores were treated by HT at 86 °C for 30 min or HPCD + HT at 20 MPa, 84-86 °C for 30 min. The HPCD + HT-treated spores are described in Table 1, and fluorescence microscopy and flow cytometry were carried out as described in Materials and Methods.

**Table 3**
Effects of high salt and glucose on the viability of untreated and HPCD + HT-treated wild-type spores that retained CaDPA and the ability of these spores to retain CaDPA upon heat treatment.[a]

| Treatments | Inactivation (%) | Viability of spores on various plates (%) | | | CaDPA content (%) | [e]aDPA release (%) |
|---|---|---|---|---|---|---|
| | | [b]LB | [c]LB + NaCl | [d]LB + NaCl + D-glucose | | |
| Untreated | 0 | 100a | 102.4 ± 5.1a | 97.6 ± 7.5a | 100a | 2.6 ± 0.8d |
| HPCD + HT, pellet | 99.83 | 100a | 8 ± 3.2b | 25 ± 4.3c | 97.9 ± 1.9a | 71.4 ± 5.5e |

[a] Untreated or HPCD + HT-treated wild-type spores that retained CaDPA as described in Table 1 were spread on LB medium plates with or without various additions, and spore viability after plates were incubated for 24–48 h at 37 °C was determined as described in Methods. Different letters indicated significant difference for all the viability, CaDPA content and CaDPA release data ($p < 0.05$). The viability and CaDPA content of untreated spores on LB medium plates was set at 100%.
[b] Normal LB plates that contain 150 mmol/l NaCl.
[c] LB medium plates with 1 mol/l NaCl.
[d] LB medium plates with 1 mol/l NaCl plus 50 mmol/l D-glucose.
[e] DPA release from spores in water treated at 85 °C for 45 min.

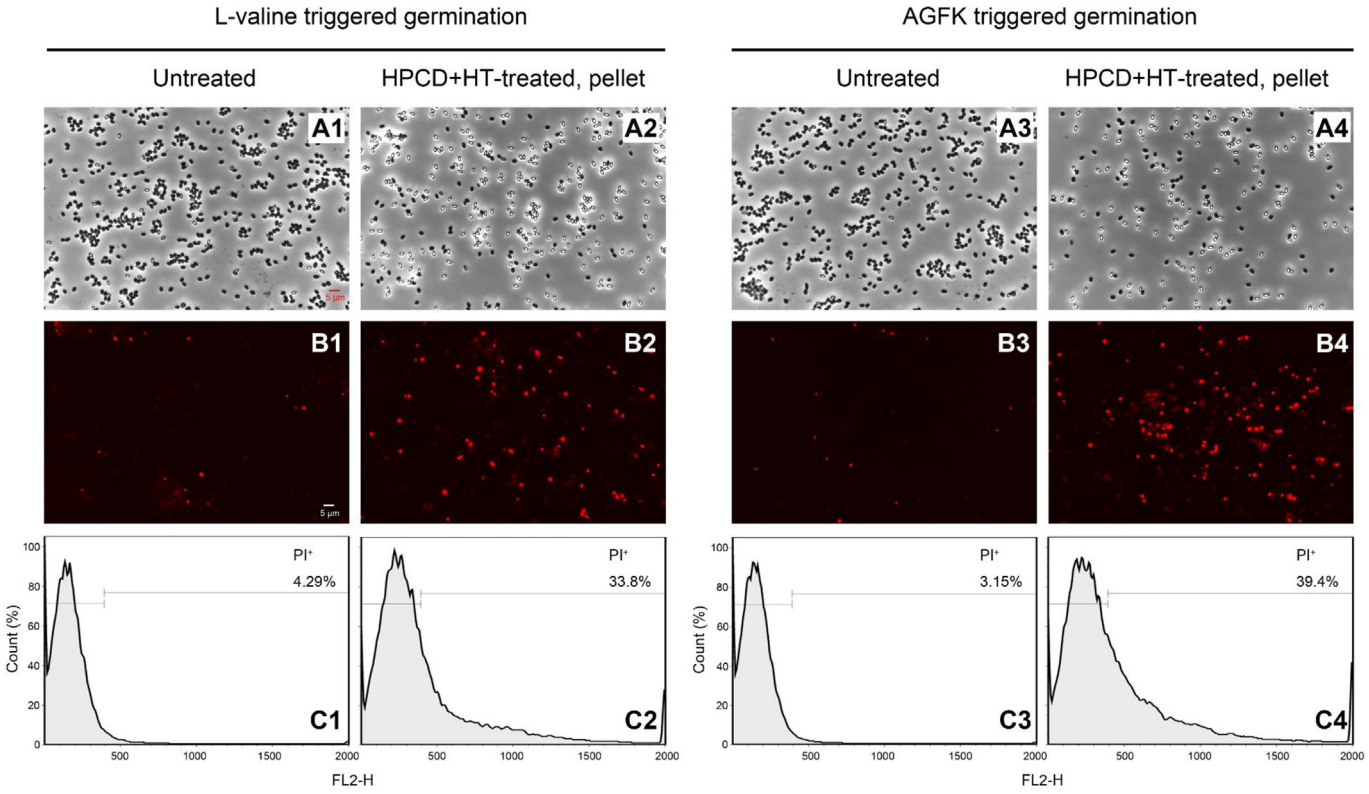


**Fig. 6.** Phase contrast photomicrographs (A1−A4), fluorescence photomicrographs (B1−B4) and flow cytometry histograms (C1−C4) of untreated and HPCD + HT-treated PS533 (wild-type) spores stained with PI after germination. Germination and microscopy were carried out as described in Materials and Methods. $PI^+$ values in panels C1−C4 indicated the percent of total spores that were stained by PI as determined by flow cytometry of ~30,000 spores as described in Materials and Methods.

### *3.5. The outgrowth defect of HPCD + HT-treated spores that retained CaDPA was due to IM damage*

Since previous work had shown that *B. subtilis* spores treated with HPCD plus peracetic acid are most likely killed by IM damage (Setlow et al., 2016), we carried out several tests to assess IM properties of HPCD + HT-treated spores that retained CaDPA. These tests to treated spores included: i) the ability of dormant spores to retain CaDPA during a normally sublethal heat treatment; ii) the ability of germinated treated spores to produce colonies on media with high salt; and iii) the staining of dormant and germinated treated spores with PI which normally does not stain dormant or germinated spores. Notably, untreated spores released only minimal amounts of CaDPA when heated for 45 min at 85 °C, but HPCD + HT treated spores retained CaDPA released large amount (Table 3). Previous work has shown that spores treated with a variety of agents that likely cause IM damage are incapable of normal colony formation on high salt media (Cortezzo et al., 2004; Setlow et al., 2016). Indeed, while untreated spores' colony formation was equal on both high and low salt LB medium plates, HPCD + HT-treated spores' colony formation was reduced 10-fold on LB plates with high salt (Table 3). As found previously with spores killed via some type of IM damage, the HPCD + HT-treated spores' low colony formation on LB plates with high salt was increased ~ 3-fold by the presence of glucose in the high salt medium, while this had no effect on colony formation from untreated spores (Table 3). These results are similar to those observed in previous studies on spore killing by a variety of treatments (Cortezzo et al., 2004; Setlow et al., 2016; Tovar Rojo et al., 2003), and have been interpreted as indicating that the IM

of these treated spores is damaged. Presumably this would also be the case for HPCD + HT-treated spores. However, whether this IM damage blocks the outgrowth of the latter damaged spores was not clear. To investigate this further, we tested the IM function of HPCD + HT-treated spores that retained CaDPA by examining the percent PI staining of germinated-treated spores (Setlow et al., 2016). Untreated and HPCD + HT-treated spores that retained CaDPA were germinated with L-valine and AGFK for 140 min, stained with PI and then examined with fluorescence microscopy and flow cytometry. For untreated spores, L-valine and AGFK germination was > 94% (Fig. 6; Table S3), but < 5% of these germinated spores were stained by PI (Fig. 6; Table S3), indicating that the IM of germinated untreated spores was intact and functional. In contrast, with dead HPCD + HT-treated spores that retained CaDPA, > 95% of L-valine and AGFK germinated spores were stained by PI (Fig. 6; Table S3), indicating that the IM of these germinated spores was damaged and could no longer maintain the membrane potential needed to prevent PI accumulation, presumably because these germinated spores plasma membrane derived from the IM has ruptured, as seen previously, thus precluding spore outgrowth (Setlow et al., 2016).

## 4. Conclusions

In summary, the most likely mechanism of inactivation of *B. subtilis* spores by HPCD + HT treatment is damage to the dormant spores' IM, such that even if the damaged spores germinated, the damaged IM led to a non-functional germinated spore plasma membrane, thus precluding spore outgrowth. Although some of HPCD + HT inactivated spores also showed some damage to GRs and/or core proteins, this damage probably occurred after spore inactivation. However, it is not clear how HPCD + HT treatment precisely damages the IM. The possibilities could be (i) the low pH resulted by HPCD causes some damage to the IM, since HPCD resulted low pH plays an important role in spore inactivation (Fig. S1), and low pH inactivated spores by damaging the IM (Setlow et al., 2002); (ii) the $CO_2$ molecules modify the IM phospholipids, which may structurally and functionally disorder the IM (Isenschmid et al., 1995; Spilimbergo, 2002), and this results in IM damage. These possibilities need to be further studied.

## Acknowledgements

This work was supported by Key Project of National Natural Science Foundation of China (NSFC) (No. 31530058), China; Key Project of Chinese Ministry of Education (No. 113011A), China; China Postdoctoral Science Foundation (No. 2018M631630), China, and Beijing Training Project For The Leading Talents in S & T (No. Z151100000315032), China.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.01.014.

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