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Resistance to and killing by the sporicidal microbicide peracetic acid

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Key words: Peracetic acid, spore, germination

Abstract

Objectives: To elucidate the mechanisms of spore resistance to, and killing by, the oxidising microbicide peracetic acid (PAA).

Methods: Mutants of *Bacillus subtilis* lacking specific spore structures were used to identify resistance properties in spores, and to understand the mechanism of action for PAA. We also assessed the effect of PAA treatment on a number of spore properties including heat tolerance, membrane integrity and germination.

Results: The spore coat is essential for spore PAA resistance, as spores with defective coats were greatly sensitised to PAA treatment. Small acid soluble spore proteins (SASPs) apparently provide no protection against PAA. Defects in spore germination, specifically to germination via the GerB and GerK, but not GerA germination receptors, as well as leakage of internal components, suggest that PAA is active at the spore inner membrane. It is therefore likely that the inner membrane is the major site of PAA's sporicidal activity.

Conclusions: PAA treatment targets the spore membrane, with some of its activity directed specifically against the GerB and GerK germination receptors.

Introduction

Some Gram-positive bacteria, such as certain *Bacillus* and *Clostridium* species, form a dormant cell type termed an endospore (spore) when encountering environmental stress such as nutrient starvation. Spores are metabolically dormant and exhibit resistance properties that are invariably greater than those of their vegetative cell type making them highly resistant to many antimicrobial treatments including extremes of temperature, radiation and many toxic chemicals.[1, 2]

In order to survive potentially long periods of metabolic dormancy where the spore is incapable of repair (reports vary greatly as to exactly how long a spore may remain viable in its dormant state),[3, 4] spores have a number of specific adaptations to minimise the damage sustained during this time. These include the outer spore coat, which has been shown to provide significant resistance against many oxidising agents,[5-8] a low core water content which contributes to the spore's resistance to some chemicals, as well as to wet heat treatment, [9, 10] and the spore-specific small acid-soluble spore proteins (SASPs) of the α/β type which saturate the spore DNA providing protection against some DNA damaging chemicals, UV radiation and heat treatments.[11-14]

Oxidising agents are increasingly finding utility in products designed for environmental disinfection. Hydrogen peroxide (liquid and vapour), chlorine dioxide, sodium hypochlorite and peracetic acid (PAA) are the active components in many such products used for hard surface and whole room disinfection and in antimicrobial wipes, and are known to be sporicidal given the correct conditions and contact times. In this report, we use the spores of *B. subtilis* to investigate

48 the mechanism of sporicidal activity, and of spore resistance, to the oxidising microbicide
49 peracetic acid.

Materials and Methods

Bacterial strains, growth conditions and spore preparation.

The *Bacillus subtilis* strains used in this study are all isogenic derivatives of strain 168. Strain PS533 is the wild-type and contains plasmid pUB110 carrying a kanamycin (Km) resistance marker. Strain PS578 (known as α^-/β^-) also contains pUB110, but lacks genes *sspA* and *sspB* which encode the two major α/β type small acid-soluble spore proteins (SASP).[15] Strain PS3394 is defective in the *cotE* gene, which has been mostly replaced by a tetracycline (Tc) resistance cassette, resulting in a defective spore coat. PS3394 also contains plasmid pUB110.[6]

All strains were routinely grown on LB medium (agar or broth; Fisher, UK) with or without antibiotic supplements (PS533/PS578: Km 10 mg/L; PS3394: Km 10 mg/L and Tc 5 mg/L). Spores of all strains were prepared on 2 × SG medium agar[16] without antibiotic selection by inoculating with 0.2 mL of a growing culture of the relevant strain (at an OD_{600} of ~1) and incubating at 37 °C for 3-5 days. All growth was then scraped from the plates and cleaned as described previously.[17]

Assessment of spore resistance.

Spore survival was routinely assessed at 25 °C and a spore titre of 10^8 cfu/mL. Microbicides used were PAA (0.025 %, 0.05 %, 0.1 % or 0.2 %) or NaOH (2 M), all prepared in dH_2O. Spore resistance to moist heat was assessed by incubating spores in water at 85 °C. After relevant contact times, samples were removed and diluted 1:100 in neutralising solution: for PAA, sodium thiosulphate, 20 g/L; for NaOH, KPO_4 buffer (50 mM, pH 7.5); for moist heat, PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, KH_2PO_4 1.4 mM) at room

temperature for a minimum of 10 min. Neutralised aliquots were serially diluted (1:10) in sterile deionised water, plated on to LB agar (without antibiotics), and incubated at 37 °C for 16-30 h before counting survivors.

For experiments preparing spore populations that had been partially inactivated (< 99 %) by PAA treatment, test solutions were neutralised in their entirety by diluting 1:10 in sodium thiosulphate (20 g/L) and incubating for 10 minutes and then filtered to harvest the spores. Samples were neutralised in fresh sodium thiosulphate (20 g/L) a further two times before washing three times in sterile PBS.

Spore recovery by lysozyme treatment.

For some experiments, spores treated with various microbicides were chemically de-coated as previously described[18] (but with incubation at 60 °C, not 37 °C) and germinated in a hypertonic medium containing lysozyme.[19]

Assaying dipicolinic acid (DPA) concentration in spores

Spores at an OD_{600} of ~ 1, with or without PAA treatment, were autoclaved to release DPA from the spore core. DPA was then assayed from the supernatant as described previously.[20] For some experiments, spores with or without PAA treatment were heated at 85 °C in a water bath for 30 minutes, and the concentration of DPA released into the supernatant assayed as described previously.[20]

Spore germination assays.

The germination of spores treated or untreated with PAA was assessed over time by monitoring the drop in OD_{600} using a Bioscreen C analyser. Spores were suspended at an OD_{600}

of ~ 1 in nutrient germinants (either 10 mM L-alanine in 50 mM Tris-HCl (pH 7.4), or the AGFK mixture composed of 12 mM L-asparagine, 13 mM D-glucose, 13 mM D-fructose and 12.5 mM KPO_4 buffer (pH 7.4))[21] and triplicate 200 µL aliquots added to the wells of the proprietary honey-comb plates used for the Bioscreen analyser. Plates were incubated at 37 °C with continuous shaking, with OD_{600} readings taken every 2 min for 300 min. At the end of 300 min, samples were removed from at least one of the replicate wells for inspection by phase contrast microscopy to ensure spores were not clumping (which can dramatically alter the OD), and to manually enumerate the germinated (phase dark) spores.

Statistical analysis

Data describing spore killing as a function of time were fitted to a Weibull microbial survival model using GinaFit, software optimized for the analysis of non-linear microbial inactivation data.[22] We then used the parameters from these models to interpolate the time required to reach a 99.9% reduction in spore viability. Due to the rapid killing of strain PS3394 by PAA treatment, it was not possible to accurately estimate values for 90 % or 99 % reductions, therefore values for 99.9 % reduction were used for all strains and at all PAA concentrations tested.

The effect of PAA treatment on DPA release from spores was compared in the software package R 3.0.1 using a Generalized Linear Model fitted with a Gaussian error and identity link function. Normal distribution of residuals was confirmed by visual examination of histograms, Q-Q plots and fitted values, before the models were refined by stepwise deletions.

Results

Resistance of *B. subtilis* strains to varying concentrations of PAA

The PAA resistance of *Bacillus subtilis* strains PS533 (WT), PS578 ($\alpha^-\beta^-$) and PS3394 ($CotE^-$) was assessed using a suspension test method. Strains PS533 and PS578 had similar PAA resistance to 0.1 % and 0.2 % PAA (Figure 1, Table 1), however PS578 showed greater resistance than did PS533 at a PAA concentration of 0.05 % (Table 1). Strain PS3394 was considerably more susceptible than strains PS533 and PS578 to PAA treatment at all concentrations tested (Table 1). Using the death times calculated for 99.9 % killing shown in Table 1, the concentration exponent (η) of PAA for each strain was calculated as 3.46, 3.53 and 1.43 for strains PS533, PS578 and PS3394 respectively. It should be noted that for strains PS533 and PS578, the kill curves show neither a significant shoulder nor a tailing effect, whereas the curves for PS3394 at all PAA concentrations tested showed a distinct tail after an initially rapid killing (Figure 1 and results not shown).

Spore recovery by lysozyme treatment

Spores treated with certain microbicides, such as NaOH, are rendered unable to germinate (most likely as a result of inactivation of the spores cortex lytic enzymes (CLE))[23] and consequently are recorded as killed using a standard testing method. However, such spores may be recovered by de-coating and treatment with lysozyme.[23] We therefore investigated the possibility that PAA treated *B. subtilis* spores could be revived by lysozyme treatment. Spores killed to 89 % or 96 % by PAA showed no recovery when de-coated and treated with lysozyme, whereas spores killed to 99.8 % by NaOH showed considerable recovery following lysozyme treatment (Figure 2), as seen in a previous study.[23]

Sensitisation to normally sub-lethal heating

It has been demonstrated previously that spores treated with various oxidising agents become sensitised to certain treatments, such as heating at 85 °C.[24] We therefore investigated the effect of heating in water at 85 °C on the survival of PAA treated spores. Untreated wild-type spores showed virtually no killing following heating at 85 °C for a period of 5 h, whereas PAA treated spores were sensitised to this treatment, as > 99 % of the population was killed after 5 h (Figure 3).

Leakage of DPA from the spore core

Spores undergoing various treatments, especially moist heat, release some/all of their DPA from the spore core.[23, 25, 26] We therefore assessed whether or not spores killed by PAA released any of their core DPA. Spores had significantly lower DPA concentrations in their core following PAA treatment when compared to untreated controls (Table 2; $F_{1,3} = 31$, $P < 0.001$), amounting to a 29 % release of DPA. We also investigated the leakage of DPA from spores, with and without PAA treatment, when heated in water at a normally sub-lethal temperature (85 °C). DPA release from PAA treated spores was significantly higher than from untreated controls ($F_{1,13} = 61.9$, $P < 0.001$) and amounted to 60 % of total core DPA (which represents much of the DPA remaining in the spore core following PAA treatment), whereas only 8 % of core DPA was released from untreated controls following heating at 85 °C (Table 2).

Germination of PAA treated spores

Spores treated with PAA were monitored for defects of germination via the nutrient germinants L-alanine or the AGFK mixture. Wild type *B. subtilis* spores treated with PAA germinated relatively normally in L-alanine (Figure 4A), with 86 % of spores germinating (as

158 measured by phase contrast microscopy, Table 2). However, germination in the AGFK mixture
159 was greatly reduced in PAA killed spores, relative to the untreated controls (Figure 4B), with
160 only 39 % of spores germinating within the 5 h experiment (Table 2).

161

Discussion

The spore coat plays a significant role in resistance to many chemicals, and especially to oxidising agents.[5, 27-29] Similarly in this study, the spore coat was responsible for a significant proportion of wild type spore PAA resistance. Where a defective spore coat is present (strain PS3394), the concentration exponent differs from the intact strains and suggests that the absence of a complete barrier exposes the spore to an alternative, more direct, killing mechanism.[30, 31]

It has been shown previously that not only do wild-type (WT) spores suffer no obvious DNA damage from treatment with PAA (as evidenced by lack of single or double strand breaks in their DNA and by the absence of auxotrophic and asporogenous mutants), but that α^-/β^- spores similarly suffer no obvious damage to their DNA.[32] In this study, a similar result has been obtained, where the WT and α^-/β^- spores both had similar resistance to PAA. This suggests that the SASPs are not a significant resistance factor against the sporicidal activity of PAA, whereas they provide significant protection against other treatments, including hydrogen peroxide and moist heat.[18, 33, 34] This investigation therefore supports the previous evidence,[32] that the sporicidal activity of PAA is not due to damage to the spore's nucleic acids, and therefore PAA likely exerts its sporicidal activity before it penetrates as far as the spore core.

Spore killing by strong alkali (NaOH) treatment is largely a result of the inactivation of spore CLEs rendering them unable to progress beyond stage I of germination, even when stimulated by nutrient germinants, as they are unable to degrade their cortex.[23, 35] However, even for NaOH treated spores, cortex degradation can be accomplished by incubation in the presence of lysozyme, allowing the spores to complete germination and outgrowth and return to vegetative

183 growth. PAA killed spores could not be recovered by lysozyme treatment, indicating that its
184 sporicidal activity is not simply the result of inactivation of the spore's CLEs.

185 Since PAA killing appeared to be neither the result of damage to spore DNA or CLEs,
186 evidence of damage to the spore's inner membrane was sough, as previous investigations have
187 indicated that treatment by other oxidising agents damage spores in this manner.[24]

188 Spore killing by moist heat, strong acid or ethanol (at elevated temperatures) is
189 accompanied by release of DPA from the spore core[23, 26] indicating the breach of spore
190 permeability barriers, thought most probably to be the inner spore membrane.[1, 36] Treatment of
191 spores with oxidising agents tends not to cause the direct release of spore DPA, although DPA
192 can be released more readily from such spores upon exposure to normally sub-lethal heat
193 treatment.[5-7, 29] Treatment of wild type *B. subtilis* spores with PAA in this study led to the
194 release of ~ 29 % of the total core DPA, indicating that PAA treatment directly altered the
195 permeability of the inner spore membrane. Furthermore, as these spores were killed to ~ 98 %
196 by PAA treatment, it was concluded that DPA release is not a function of spore killing, as some
197 spores are apparently dead and yet have released no DPA.

198 Most of the DPA remaining in the spore core following PAA treatment was released
199 when spores were incubated at 85 °C, which caused little DPA release from untreated spores.
200 That PAA treated spores leak DPA from their core on normally sub-lethal heating strongly
201 suggests that the membrane has suffered damage which, whilst not overwhelming the
202 permeability barrier of the membrane entirely, has weakened it to such an extent that it is
203 breached upon heating. Presumably, given the observation above that DPA release does not
204 correlate precisely with PAA kill, such membrane damage is also severe enough that these

spores are unable to complete outgrowth and are non-viable, although they can, to a great extent, still germinate.

Previous studies have shown that altering either the level of saturation of membrane fatty acids or the lipid composition of the spore membrane have minimal effects on spore resistance to oxidising agents,[24, 37] although this does not rule out their being a target for oxidative damage and therefore a possible site of DPA release from the spore core. It has also been suggested that membrane proteins, such as the nutrient germination receptors (GRs), or the SpoVA proteins which are thought to reside within the spore membrane and are very likely involved in release of DPA (in the form of a 1:1 chelate with calcium ions) from the spore during germination[38, 39], could also be targets for oxidative damage.

Germination by nutrient germinants in *B. subtilis* is triggered via GRs, each comprising three individual proteins, located in or at the spore inner membrane.[35] The GerA receptor recognises and binds L-alanine or L-valine only, where as the GerB and GerK receptors apparently interact to allow germination via a mixture of AGFK.[35] PAA treatment altered *B. subtilis*' response to germination via nutrient germinants, both reducing the rate at which spores germinated and altering the number of spores completing germination within the observed time frame. Whilst it is recognised that spores treated with oxidising agents often germinate poorly in response to some germinants,[5-7, 29, 40, 41] this study clearly shows that PAA treatment caused a more pronounced alteration of spore germination via the AGFK mixture compared to germination with L-alanine. These results suggest that i) PAA treatment is preventing germinant molecules from accessing the GR in the inner membrane or ii) PAA is somehow altering the ability of GRs to respond to their trigger molecules.

It seems unlikely that germinants are not able to reach their receptors following PAA treatment, at least for L-alanine, as germination proceeded relatively normally with this germinant. Therefore, it would appear that PAA is damaging some/all of the GerB and GerK GRs such that they no longer respond to their trigger molecules, or at least respond more slowly. This would be analogous to the situation found in superdormant (SD) spores, where spores having fewer GRs for a given germinant germinate only very slowly.[21, 42] As for the reasons why GerB and GerK are apparently more susceptible to PAA treatment than GerA, there could be several explanations: i) there are more of GerB and GerK present in the inner membrane, relative to GerA, thus making GerB/K more likely to be damaged by PAA simply by frequency of presence, ii) the amino acid make up of GerB/GerK makes them more susceptible to oxidation by PAA than that of GerA, and/or iii) that a greater portion of the GerB/GerK receptor complex is exposed at the surface of the membrane, compared to GerA.

In a recent study, it was identified that GerA is in fact the most abundant GR in the spore with ~1,100 molecules/spore, with GerB and GerK both present at ~700 molecules/spore.[43] However, taking into account that both GerB and GerK are required for germination with AGFK, we can take it that there are a total of ~1,400 molecules/spore available as a target for PAA, which is slightly higher than the 1,100 GerA molecules. Whether this difference is sufficient to cause the differing response in germination seen in this study is not known, but it is also worth noting that there are other protein molecules present in the spore inner membrane at far higher number than those of the germination receptors,[43] which could presumably also suffer damage from oxidising agents such as PAA, but were not assessed in this study. Indeed, a recent study showed that treatment of spores with hypochlorite or hydrogen peroxide caused a lengthening of the time taken for spores to release their DPA during germination, perhaps indicating some

damage to the SpoVA proteins in the inner membrane which are involved in DPA release during germination.[40]

SD spores have been shown to possess greater resistance to moist heat than do normal spores, although this was thought to be a reflection of the lower core water content of SD spores rather than to any other spore properties.[44] It would be interesting to characterise the oxidising agent resistance properties of SD spores, which have been shown to contain lower levels of nutrient GRs,[21] as one would expect that SD spores for AGFK germination may be more resistant to PAA, assuming that damage to GerB/GerK contributes to the sporicidal activity of PAA. A recent study of SD spores under germination with the non-nutrient germinant dodecylamine found that whilst these SD spores did contain lower GR levels, they were no more resistant to treatment with sodium hypochlorite than normal wild type spores, however this was not the primary focus of the investigation and was not studied in detail.[45]

Whilst sequence data is available for the germination receptors in *B. subtilis*, and other spore formers, there is limited information in the literature regarding their structures and/or membrane topology.[46-48] It is highly probable (based on sequence data) that the A and B GR subunits are integral membrane proteins, whilst the C subunit is not an integral membrane protein but is located on the membrane periphery.[46, 48, 49] Further investigation of this data may offer some suggestion as to the apparently different susceptibility of the different GRs to the activity of PAA.

That PAA is acting to disrupt nutrient germination in *B. subtilis* seems likely, although whether this activity is the major source of, or even contributes to the sporicidal activity of this microbicide remains unclear. It is also unclear whether activity against membrane-associated

proteins alone would be sufficient to destabilise the membrane such that it can no longer function when the spore undergoes germination, thus preventing outgrowth.

This study suggests that PAA kills spores by causing damage to the inner spore membrane, as is thought to be the case for other oxidising agents. It appears also that the membrane proteins GerB and GerK are damaged by PAA treatment, possibly contributing to the sporicidal activity of PAA.

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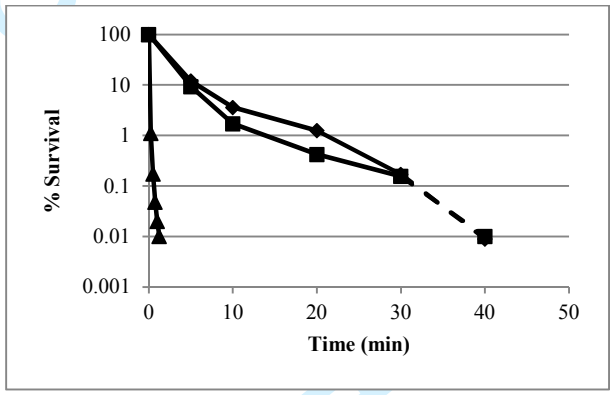
Transparency declarations: Peter Burke, Gerald McDonnell and J. Spencer Schwartz are employees of STERIS Corporation.

399 A)



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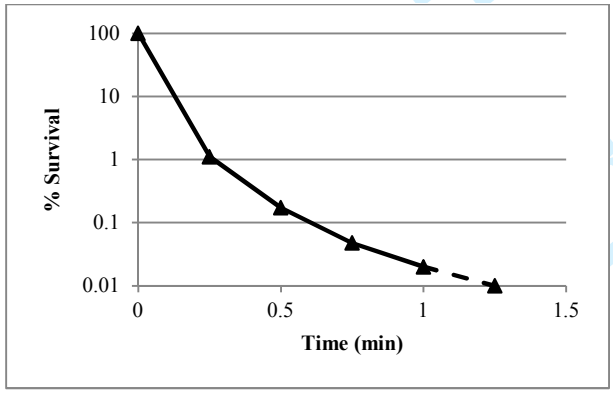
401 B)



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403 Figure 1. Killing of *B. subtilis* spores by 0.1 % PAA. (a) PS533 (wild type; filled diamonds),
404 PS578 ($\alpha^-\beta^-$; filled squares) and PS3394 (*cotE*$^-$; filled triangles). (b) killing of strain PS3394 as
405 in a, but with shortened time axis. Data points represent mean values from two experiments.
406 Dashed line represents samples that fell below the level of detection at the indicated time point.

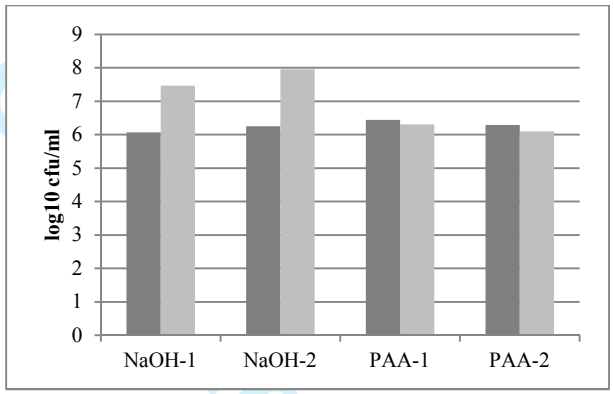


Figure 2. Recovery of spores, with/without microbicide treatment, by incubation with lysozyme. Spores were treated with NaOH or PAA giving kill (%) of 99.8 for NaOH-1 and NaOH-2, 89.4 for PAA-1 and 96.3 for PAA-2. Microbicide treated spores were incubated with (light shading) or without (dark shading) lysozyme.

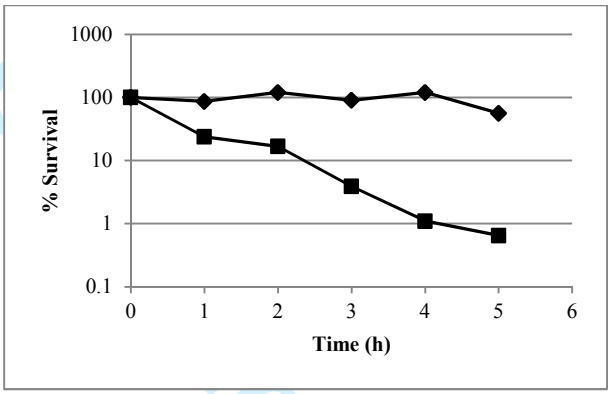
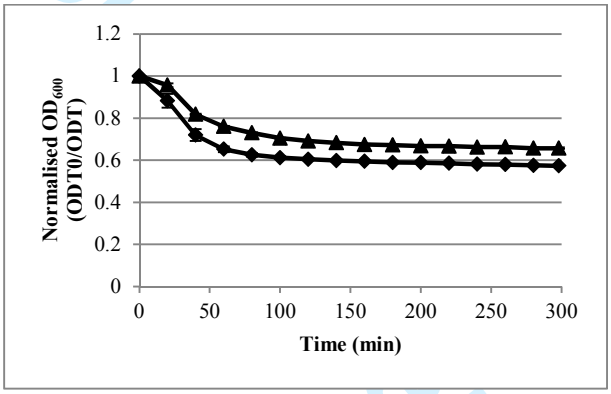


Figure 3. Survival of WT (PS533) spores, with and without PAA treatment giving 97.7 % spore killing, when heated in water at 85°C. Data points represent mean values from two experiments. Untreated control (filled diamonds), PAA treated (filled squares).

A)



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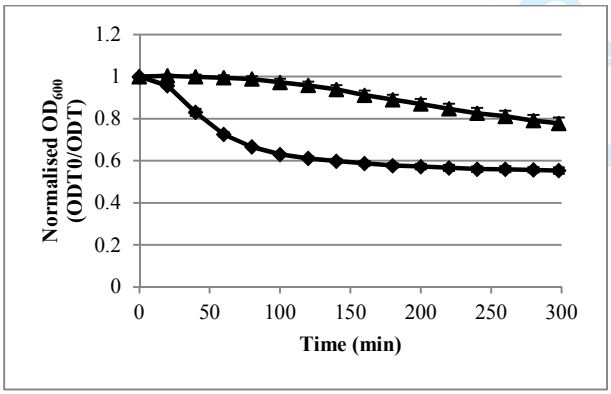

Figure 4. Germination of *B. subtilis* PS533 spores with and without PAA treatment. Spores were germinated in A) L-Alanine and B) AGFK mixture. Untreated control (filled diamonds), PAA killed (98.8%; filled triangles). Data points represent mean values from two (control) or three (PAA killed) experiments and include representatives from two independent parent spore stocks. Error bars represent standard error of the mean and are obscured by the symbols for some data points. Note that not all data points recorded are presented in this figure.

438 Table 1. Calculated times to achieve 99.9 % kill of *B. subtilis* spores at various concentrations of

439 PAA.

PAA Concentration	Calculated time (min) to give 99.9 % kill of *B. subtilis* strains:		
(%)	PS533	PS578	PS3394
0.025	NT*	NT*	6.8
0.05	182.48	233.10	1.8
0.1	36.39	32.74	0.62
0.2	1.51	1.74	0.36

440 *NT – not tested.

441

442 Table 2. Various properties of *B. subtilis* PS533 spores with and without PAA treatment.

Treatment	Germination (%) after 300 min		DPA** (mg/L) released by:	
	L-Ala	AGFK	Autoclaving	Heating at 85 °C
None	99	100	24.28 ± 0.55***	1.94 ± 0.33
PAA*	86	39	17.18 ± 0.67	14.66 ± 0.98

443 * Spores treated with PAA to achieve < 99 % killing. ** data represent mean values ± standard
444 error of the mean (n = 15). *** This value was used as 100 % of spore DPA.

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