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Expression of the 2Duf protein in wild-type *Bacillus subtilis* spores stabilizes inner membrane proteins and increases spore resistance to wet heat and hydrogen peroxide

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Abstract

Aims: This work aimed to characterize spore inner membrane (IM) properties and the mechanism of spore killing by wet heat and H_2O_2 with spores overexpressing the 2Duf protein, which is naturally encoded from a transposon found only in some *Bacillus* strains with much higher spore resistance than wild-type spores.

Methods and Results: Killing of *Bacillus subtilis* spores by wet heat or hydrogen peroxide (H_2O_2) was slower when 2Duf was present, and Ca-dipicolinic acid release was slower than killing. Viabilities on rich plates of wet heat- or H_2O_2 -treated spores +/- 2Duf were lower when NaCl was added, but higher with glucose. Addition of glucose but not Casamino acids addition increased treated spores' viability on minimal medium plates. Spores with 2Duf required higher heat activation for germination, and their germination was more wet-heat resistant than that of wild-type spores, processes that involve IM proteins. IM permeability and lipid mobility were lower in spores with 2Duf, although IM phospholipid composition was similar in spores +/- 2Duf.

Conclusions: These results and previous work suggests that wet heat and H_2O_2 kill spores by damaging an IM enzyme or enzymes involved in oxidative phosphorylation.

Significance and impact of study

This work provides evidence consistent with a hypothesis for how wet heat and H_2O_2 kill spores.

Keywords: bacterial spores, *Bacillus*, antimicrobials, disinfection, metabolism

Introduction

Spores of some Firmicute species play major roles in food spoilage and food-borne illness and can cause serious human diseases (Setlow and Johnson 2019). Two important properties in these spores' deleterious effects are: (1) metabolic dormancy enabling spore survival for years without metabolites and without the need for ATP; and (2) resistance to all manner of treatments that kill growing cells, including wet heat, dry heat, UV and γ-radiation, and chemicals (Setlow 2016).

Features of spores crucial in their dormancy and resistance include spores' very different structure from that of growing cells (Setlow 2016, 2019, Setlow and Johnson 2019). From the inside out there is the spore core, analogous to a cell's protoplast and containing DNA, RNA, and enzymes for macromolecular synthesis, energy metabolism, and spore-specific proteins that saturate and protect DNA from damage. Notably, core metabolic enzymes are inactive in spores (Ghosh et al. 2015). Surrounding the core is the IM, the location of many proteins essential in spore germination and oxidative phosphorylation (Zheng et al. 2016, Setlow et al. 2017, Gao et al. 2021). The IM is surrounded by two layers of peptidoglycan (PG), a thin germ cell wall with a structure apparently identical to that of growing cell PG, and an outer, thicker cortex layer with cortex PG-specific modifications. The cortex PG is degraded in spore germination by two redundant cortex-lytic enzymes (CLEs) that recognize cortex PG-specific modifications, thus allowing core and IM expansion (Setlow et al. 2017). One CLE, SleB, is in the outer rim of the cortex and the IM, while the second CLE, CwlJ, is reported only in the coat/cortex fraction of disrupted spores (Bagyan and Setlow 2002, Chirakkal et al. 2002, Setlow et al. 2017). Next is the outer membrane (OM), which while important in spore formation, may have no crucial role in dormant spores. Outside the OM is the coat composed of several layers, a crosslinked structure with over 70 spore-specific proteins (Driks and Eichenberger 2016). The coat is somewhat rigid, except for the outer layers, and this may be important in restricting outward expansion of the cortex during its formation, exerting pressure on the core and IM and forcing some IM into the core and extrusion of some core water (Paidhungat et al. 2000, Laue et al. 2018). The outermost layer in spores of some species is an exosporium, and in *Bacillus subtilis* is a

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crust layer, but these layers may have no roles in spore dormancy or resistance.

Properties of spores' core, IM, and coat playing major roles in spore dormancy and resistance include the core's very low water content, as low as 25% of wet weight and 35% of wet weight in spores of the model spore former *B. subtilis* (Gerhardt and Marquis 1989, Paidhungat et al. 2000). The core's low water content is due in large part to the cortex, and the core's accumulation of a 1:1 chelate of Ca^{2+} and dipicolinic acid (CaDPA) late in sporulation to ∼25% of core dry weight leads to further loss of core water. The core's low water content plays a major role in spore dormancy, as at least one soluble protein is immobile in the core (Cowan et al. 2003) and is very important in spore resistance to wet heat, which is inversely related to the core water content (Gerhardt and Marquis 1989). Finally, spore DNA is saturated with spore proteins that provide DNA resistance to radiation, heat, and damaging chemicals (Setlow 2016).

A second layer crucial in spore resistance is the IM, and while IM lipid composition is very similar to that of a growing cell membrane (Griffiths and Setlow 2009), its properties are not. Thus, a lipid probe in the IM of spores of several *Bacillus* species is largely immobile, while it is much more mobile in germinated spores or growing cells (Cowan et al. 2004), and IM lipids in spores of several *Clostridium* species have low fluidity, with high levels of lipid order (Hofstetter et al. 2012). *Bacillus* spore IM permeability is also lower than that of a growing cell—even to water or small hydrophobic compounds such as methylamine (Swerdlow et al. 1981, Sunde et al. 2009, Kaieda et al. 2013, Knudsen et al. 2015). Overall, it seems likely that IM permeability and/or rigidity plays a major role in spore resistance to chemicals that must cross the IM to damage either core DNA or proteins (Kanaan et al. 2021). In addition, previous work found that oxidative damage to spores' IM caused spore wet heat resistance to decrease, and large alterations in *B. subtilis* spore IM phospholipid content greatly decreased spores' resistance to wet heat, further indicating a major potential role for the IM in these spore resistance properties (Cortezzo et al. 2004, Griffiths and Setlow 2009).

An additional IM effect on spore resistance is exerted by the 2Duf protein, which is encoded on a transposable element originally identified in very wet heat resistant spores of *Bacillus* species closely related to *B. subtilis* (Berendsen et al. 2016, Krawczyk et al. 2016). The 2Duf protein also increases spore resistance to chemicals that must cross the IM to damage DNA or core proteins (Kanaan et al. 2021). 2Duf has been predicted to be an IM protein, although this has not yet been shown directly. The DPA in the core and the coat itself also have roles in IM permeability, and loss of the *B. subtilis* spore coat, which increases IM permeability to water (Knudsen et al. 2015) also decreases spore wet heat resistance (Ghosh et al. 2008). Loss of both spore CaDPA and the coat results in an IM with very high permeability, and more rapid killing by chemicals that must cross the IM to damage core components (Mokashi et al. 2020, Kanaan et al. 2021).

There has been much work on mechanisms of spore killing (Christie and Setlow 2021), a subject of much applied interest. Work in this communication characterizes several aspects of spores, spore resistance and killing, including how changes in the spore IM due to 2Duf lead to increased spore resistance to wet heat and hydrogen peroxide (H_2O_2). This work provides evidence that protection of IM enzymes essential in ATP generation is crucial in decreasing spore killing by wet heat and H_2O_2, and that damage to such enzymes is likely how these agents kill spores.

Materials and methods

Strains used and spore preparation and purification

The spore formers used in this work are strains of *Bacillus subtilis* 168. Three strains (Berendsen et al. 2016, Kanaan et al. 2021, Luo et al. 2021) are isogenic and are PS4461, a wild-type (wt) strain, PS4462 that is spectinomycin resistant (Sp^r) and contains an operon called *spoVA*^{2mob} that carries the *2duf* gene transcribed in the developing spore under control of the sigma G subunit for RNA polymerase, which transcribes forespore-specific genes in sporulation (Arieta-Ortiz et al. 2015); PS4465 is identical to PS4462 but lacks the *2duf* gene. PS4462 spores' wet heat resistance is much higher than that of PS4461 spores, and PS4465 spores wet heat resistance is only slightly higher that of PS4461 spores (Luo et al. 2021). Two other isogenic strains are: (i) PS832 wt, a 168-laboratory strain, and (ii) PS533 (also wt) (Setlow and Setlow 1996), PS832 that carries plasmid pUB110 providing resistance to kanamycin (Km^r).

All strains are from the Setlow laboratory and spores 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). After 2–3 d of incubation, spores were scraped from plates into 4°C water and purified by multiple rounds of sonication and centrifugation over 2–4 d, with a final purification step of centrifugation through a solution of ∼50% Histodenz, in which CaDPA-replete wt spores pellet and debris floats (Setlow 2019). All spores used in this work were >98% free of growing or sporulating cells, germinated spores, or visible debris, and were stored at 4°C protected from light at an optical density at 600 nm (OD_{600}) of ∼10. Coats were removed from spores at an OD_{600} of 50 in a decoating solution in water of 8 mol l^{-1} urea–50 mmol l^{-1} Tris–HCl (pH 8.0)—10 g l^{-1} SDS–50 mmol l^{-1} dithiothreitol–10 mmol l^{-1} EDTA (Bagyan et al. 1998). After incubation for 45 min at 37°C, the spores were centrifuged, the pellet suspended to 1 ml in decoating solution without EDTA and incubated for an additional 45 min at 37°C. The spores were washed six times by centrifugation with 1 ml of 10 mmol l^{-1}–Tris–HCl (pH 8)–10 mmol l^{-1} EDTA–150 mmol l^{-1} NaCl, and then washed with and stored in water at 4°C.

Preparation of di-4-ANEPPS-labeled spores and analysis of dye's mobility

Preparation of pyridinium, 4-(2-(6-(dibutylamino)-2-naphthalenyl) ethenyl)-1-(3-sulfopropyl)-, hydroxide, inner salt (di-4-ANEPPS)-labeled spores' inner membrane (IM) was in liquid 2 × SG medium at 37°C as described (Cowan et al. 2004), with 2 μM dye added late in log-phase growth, and spores were purified as described above. The purified labeled spores were analysed by fluorescence redistribution after photobleaching (FRAP), essentially as described previously (Cowan et al. 2004), except that diffusion coefficients (D) and mobile fractions were determined by comparing experimental results to simulated results using a 3D computational model of the FRAP experiment developed in the Virtual Cell (VCell)

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software (Schaff et al. 1997, Cowan et al. 2012), The VCell model "*Bacillus* Spore Membrane FRAP Analysis" user dfairchild can be accessed within the VCell software available at https://vcell.org. This analysis accounted for bleaching during monitoring and unquenching after bleaching, both determined in control experiments, as well as diffusion during the redistribution after photobleaching. Average values of D and the mobile fraction from five individual spores were determined (Table 2).

Spore IM permeability and core pH measurements

Determination of rates of permeation of a molecule across the IM and into the spore core, as well as core pH, was by measuring radioactive methylamine incorporation into intact and decoated spores as described (Cortezzo et al. 2004). Spores of various strains (~15 mg dry weight ml^{-1}) in 200 mmol l^{-1} Tris–HCl (pH 8.8) were incubated at 24°C with 5 μmol l^{-1} ^{14}C-methylamine (Moravek Biochemicals, Brea, CA, USA) (~2.5×10^5 dpm ml^{-1}). At various times, 1 ml samples were rapidly passed through a 0.22-micron centrifuge filter, the filtrate saved, the filter washed with two 500 μl aliquots of 4°C 200 mmol l^{-1} Tris–HCl buffer (pH 8.8), and 500 μl of 50 g l^{-1} trichloroacetic acid was added above the filter. After incubation overnight to elute ^{14}C-methylamine, the filter was centrifuged to collect the eluate, and 500 μl of the eluate, 500 μl of the initial filtrate and washes were added individually to 5 ml scintillation fluid and samples were counted in a scintillation counter. Spore counting in a Petroff-Hauser chamber was used to correct the levels of ^{14}C-methylamine uptake for slight variations in spore numbers between samples. Values of the pH in the spore core were calculated from the maximum accumulation of ^{14}C-methylamine relative to that of PS832 spores, for which the spore core pH was determined by this method as ~6.4 (Swerdlow et al. 1981), consistent with values determined with a pH sensitive dye (Magill et al. 1994).

Measurement of spore killing and germination

Wet heat treatment of spores was carried out with spores in water at various temperatures given in Tables or Figure legends with ~1.5×10^8 spores ml^{-1}. At various times, 100 μl aliquots were diluted 10-fold in 4°C sterile water, and then serially diluted 10-fold in sterile 23°C water. Aliquots (10 μl) were spotted in duplicate in a grid on Luria Broth (LB) medium plates (Paidhungat et al. 2000) that were incubated overnight at 30°C, and then at 37°C until no more colonies appeared, and colonies were counted. In some experiments spores were spotted on LB plates containing 1 mol l^{-1} NaCl or 20 mmol l^{-1} glucose. In other experiments, treated spores were spotted on Spizizen's minimal medium (Spizizen 1958) plates with 0.1% casamino acids +/- 30 mmol l^{-1} glucose, or with 30 mmol l^{-1} glucose along with 100 μmol l^{-1} L-alanine to trigger spore germination. The only carbon source in Spizizen's minimal medium is citrate, and spores of a number of *Bacillus* species including *B. subtilis* do not have a complete tricarboxylic acid cycle (Kornberg et al. 1968, Setlow et al. 1977, Swarge et al. 2018). In some cases, aliquots of heated spores were centrifuged and DPA in the supernatant fluid was determined by measurement of Tb-DPA fluorescence as described below. Total spore DPA was measured similarly on dormant spore samples that were extracted by boiling for 30 min in water (Luo et al. 2021). H_2O_2 killing of spores was carried out with 10% H_2O_2 at 23°C as described previously, as was dilution of incubations 1/10 in a catalase solution for H_2O_2 inactivation (Kanaan et al. 2021), and spore viability and DPA release were assessed on LB or Spizizen's minimal medium plates with various additions, as described above.

Spore germination was preceded by heat activation at an elevated temperature and then cooling, and spores were germinated at ~8×10^7 ml^{-1} and 37°C in 200 μl of 25 mmol l^{-1} K-Hepes buffer (pH 7.5), usually with 10 mmol l^{-1} L-valine and +/- 50 μmol l^{-1} $TbCl_3$, and Tb-DPA fluorescence (Yi and Setlow 2010) or conversion of spores to phase dark forms in a phase contrast microscope were determined. If phase contrast microscopy was used to assess germination, $TbCl_3$ was omitted from germination incubations. In an additional set of experiments, spores were incubated in water at various temperatures for various times and then germinated with either L-valine or with 10 mmol l^{-1} each of L-asparagine—D-glucose—D-fructose—KCl (AGFK). In another experiment, spores were incubated at 93°C or 98°C for various times, cooled, germinated for 90 min at 23°C in 40 mmol l^{-1} CaDPA made to pH 7.4 with Tris base, and germination was assessed by determining the percentage of spores that had turned phase dark using phase contrast microscopy, with 60–70 individual spores examined at each time point.

Quantitation of phospholipid levels in spores and vegetative cells

Procedures for extracting phospholipids from spores generally followed a protocol used earlier (Griffiths and Setlow 2009), except that decoating was by the urea-SDS method described above, and the decoated spores were lyophilized. The dry decoated spores were broken by ten 1-min periods of shaking with a steel ball in a bead beater with glass beads as the abrasive as described previously (Bagyan et al. 1998), and cells were grown in LB medium to an OD_{600} of ~3 and washed with a room temperature solution of 150 mmol l^{-1} NaCl, 25 mmol l^{-1} KPO_4 buffer at pH 7.4 and lyophilized, but only shaking for five 1-min periods. Phospholipids were extracted from disrupted cells or spores (Bligh and Dyer 1959) and extracts were dried and held at −20°C. Samples were dissolved in 0.5 ml of methanol and 70 μl transferred to an autosampler vial. Samples were run on an Avantor ACE (Excel 2 C18, 75 × 2.1 mm) column with a flow of 0.4 ml min^{-1}. Solvent A was acetonitrile: water (60:40, v/v) and Solvent B was isopropanol: acetonitrile (90:10, v/v), both supplemented with 10 mmol l^{-1} ammonium formate and 0.1% formic acid. The solvent gradient was 0–1.8 min, 70% solvent A/30% solvent B, 1.8–8 min: linear gradient to 100% solvent B, 8–8.2 min: 70% solvent A/30% solvent B. The mass spectrometer (Sciex 500) was run in multiple reaction monitoring (MRM) negative ion mode with a curtain gas pressure of 30 psi, ion source gas 1 of 50 psi, ion source gas 2 of 60 psi and an ionspray voltage of −4500 V. The parameters for the MRM analysis are given in Table S1. Peaks were integrated electronically and ion abundances for each compound were used to calculate the percentage of each lipid class in the samples.

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Table 1. *Effects of additions to recovery plates on spore viability after wet heat or H_2O_2 treatment.

Time-min	PS4461			PS4462		
	Additions to plates					
	none	NaCl	glucose	none	NaCl	glucose
	Spore survival—%					
0	100	95	105	100	93	97
60	7	1	27	-	-	-
150	-‡	-	-	3.5	0.7	9
180	-	-	-	0.8	0.15	2.5
			H_2O_2			
0	100	98	97	100	103	105
30	14	0.08	60	-	-	-
60	0.04	0.002	4.3	30	0.1	75
90	-	-	-	0.5	0.007	9

*Spores of strains PS4461 (wild-type) and PS4462 (with 2Duf) were incubated in water at 93°C (PS4461) or 98°C (PS4462) or at 23°C with H_2O_2, at various times aliquots were diluted (with catalase for H_2O_2-treated samples), and 10 μl aliquots of dilutions spotted on LB medium plates with no additions, 1 mol l^{-1} NaCl, or 20 mmol l^{-1} D-glucose, plates incubated and colonies counted as described in Material and methods section. Values are averages of duplicate determinations with deviations ≤15%. ‡—not tested.

Results

Heat and H_2O_2 killing of spores +/- 2duf, and properties of treated spores

As found with spores that lack *spoVA*^{2mob} (Coleman et al. 2007), wet heat-treated PS4461 (wt) spores lost viability more rapidly than DPA release (Fig. 1A). While PS4462 spores with 2Duf were more wet heat resistant than PS4461 spores, DPA release from wet heat-treated PS4462 spores was also slower than spore killing (Fig. 1B; note different scales on vertical axes in panels A and B). The PS4462 spores also showed higher H_2O_2 resistance compared to PS4461 spores, and spores of both strains released ≤4% DPA release at all levels of spore killing (Fig. 1C). Spore killing by wet heat or H_2O_2 was much faster than DPA release, and with ≤4% of DPA release at any time points and ≥99.5% killing at the longest time points examined, clearly spore killing by these agents was not due to major disruptions in spores' IM.

Previous work found that spore treatment with wet heat or H_2O_2 damaged spores, with the damaged spores' viability very dependent on the specific composition of the recovery medium. Stressful media such as with a high salt gave lower viabilities, and richer media with added glucose gave higher viabilities (Cortezzo et al. 2004, Coleman et al. 2007, Coleman and Setlow 2009). This was also true with wet heat-treated PS4461 and PS4462 spores, as LB plates with 1 M NaCl gave 5- to 10-fold lower viabilities than LB plates with no additions, while LB plates with glucose gave 3- to 4-fold higher viabilities (Table 1). The recovery media's effects on H_2O_2-treated spores were even larger than with wet heat-treated spores (Table 1). These results were interpreted previously as suggesting that a major defect in wet heat-treated spores was damage to one or more enzymes essential for ATP synthesis in spore outgrowth. While wet heat-treated spores often germinated relatively normally, the great majority of the germinated spores accumulated minimal levels of ATP or reduced FMN (Melly et al. 2002, Coleman et al. 2007).

To further explore the possibility that wet heat and H_2O_2 kill spores by damage to an enzyme essential for ATP generation, we used *B. subtilis* wt strain, PS533, which is prototrophic for all amino acids, and examined effects of various additions to Spizizen's minimal medium plates with only citrate and salts on recoveries of wet heat or H_2O_2 treated spores (Fig. 2A and B). Glucose addition was most effective in increasing spore recoveries on minimal medium plates, albeit with a small amount of alanine also added to ensure spore germination. Notably, addition of Casamino acids were less effective than glucose plus 100 μmol l^{-1} L-alanine. These findings suggest that spore damage by wet heat or H_2O_2 is to an enzyme of ATP generation from amino acids but not from glucose. Good candidates for the damaged enzyme or enzyme system in wet heat/H_2O_2-treated spores are thus enzymes of oxidative phosphorylation, as glucose catabolism can generate ATP by glycolysis and regenerate NAD by converting pyruvate to lactate. However, if oxidative phosphorylation is unable to deal with large amounts of electrons on NADH, as may come from amino acid catabolism, ATP generation is compromized. Notably, enzymes of oxidative phosphorylation are invariably membrane associated, and have been found in spores' IM (Zheng et al. 2016, Gao et al. 2021).

Heat activation and effects of wet heat on germination of spores +/- 2duf

The results in the previous section suggested that 2Duf changes PS4462 spores' IM properties to better protect IM proteins against wet heat and H_2O_2. Indeed, spores with higher wet heat resistance commonly have more stringent requirements for heat activation to obtain effective germinant receptor (GR)-dependent spore germination (Krawczyk et al. 2017). In contrast, GR-independent spore germination is unaffected by heat activation (Setlow et al. 2017). The ability of a sublethal heat treatment to greatly increase rates of GR-dependent spore germination has been known for years and is likely due to effects on GRs, as different GRs in the same strain can have different heat activation requirements (Luu et al. 2015); notably, GRs are in the IM (Setlow et al. 2017). To directly compare effects of 2Duf on GRs' heat activation requirement we examined *B. subtilis* PS4461 and PS4462 spores' germination with AGFK that requires the GerB and GerK GRs and has a stringent heat activation requirement (Luu et al. 2015) (Fig. 3A–C). This work found that PS4461 and PS4462 spores' AGFK germination were maximal with 80°C heat activation for ~120 and ≥210 min, respectively

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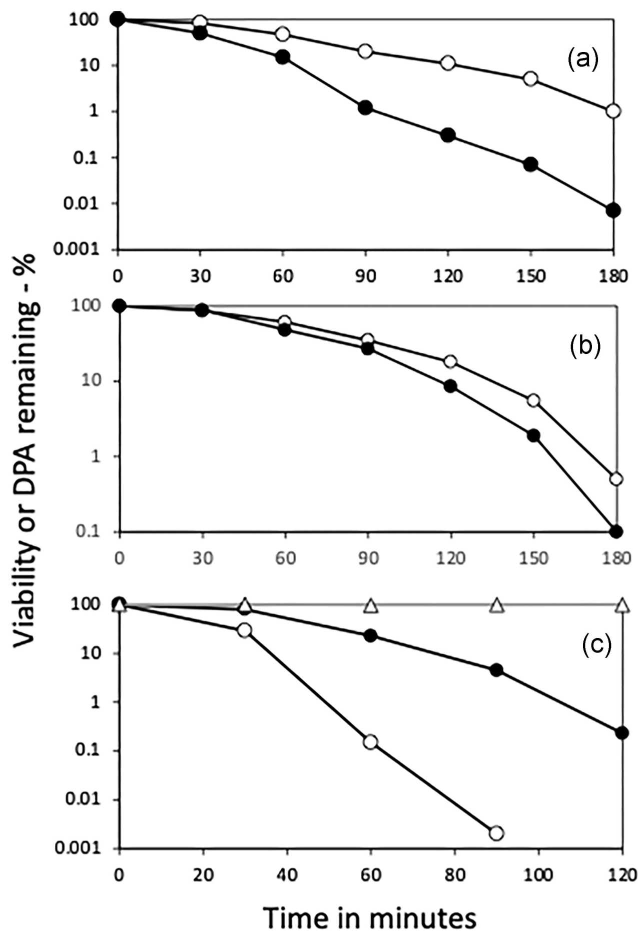

Figure 1. (A–C) Viability and DPA remaining during heat or H_2O_2 treatment of spores with and without 2Duf. Spores of strains (A) PS4461, (B) PS4462, and (C) PS4461 and PS4462 at $\sim 10^8$ ml^{-1} in water were incubated at (A and B) 90°C (PS4461) or 98°C (PS4462) or (C) in H_2O_2 at 23°C. Aliquots were serially diluted in cold water without (A and B) or with catalase (C), 10 μl aliquots spotted in duplicate on LB medium agar plates, plates were incubated and colonies were counted, as described in Material and methods section. Aliquots (0.5 ml) were taken at various times, centrifuged, and DPA quantitated in the supernatant fluid and pellet as described in Material and methods section. All values are +/- ≤12%. The symbols in (A and B) are: ○, DPA remaining; •, viability, and in (C) are ○, •–viability of PS4461 and PS4462 spores, respectively, and △—DPA remaining in PS4462 spores; slightly less DPA was released from PS4461 spores (data not shown).

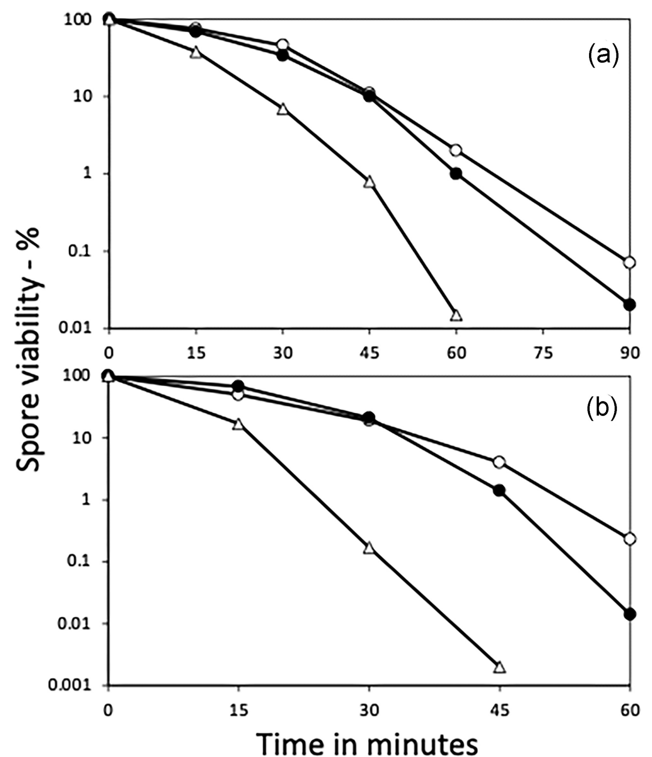

Figure 2. (A and B) Spore viability on minimal medium plates with various additions after treatment with (A) 93°C or (B) H_2O_2. PS533 spores (wt) were treated, cooled on ice or H_2O_2 eliminated, and spore viability determined on Spizizen's minimal medium plates with various additions as described in Material and methods section. The symbols for additions to recovery plates are: 0.1% Casamino acids–△; 0.1% Casamino acids and 30 mmol l^{-1} glucose–○; and 30 mmol l^{-1} glucose plus 100 μmol l^{-1} L-alanine–•. All values are +/- ≤ 16%.

(Fig. 3C). However, even with optimal heat activation, PS4462 spores' AGFK germination was slower than of optimally heat-activated PS4461 spores, as reported previously (Krawczyk et al. 2016, 2017) (Fig. 3A–C).

While heat activation increases spore germination rates markedly, if too long or at too high a temperature, this can kill spores, and slow or eliminate germination (Wen et al. 2022). Since GRs as well as one CLE, SleB (Chirakkal et al. 2002) are IM proteins, we examined if extended wet heat treatment had different effects on germination of spores +/- 2Duf (Fig. 4A and B). In one experiment, effects of heat treatment on PS4461 and PS4462 spores' ability to complete spore germination triggered by L-valine via the GerA GR was measured, with completion of germination monitored by the percentage of spores that became dark in phase contrast microscopy (Fig. 4A). Clearly spores with 2Duf were more able to tolerate longer times at high temperatures without losing the ability to complete germination, consistent with higher stability of the IM GerA GR, the IM SpoVA protein channel for CaDPA release and IM SleB in spores with 2Duf. In a second experiment (Fig. 4B), PS4461 and PS4462 spores were wet heat treated for various times, cooled, and incubated with CaDPA at 23°C to trigger spore germination via activation of the CLE CwlJ (Setlow et al. 2017). Complete germination of PS4462 spores with exogenous CaDPA requires endogenous CaDPA release and thus IM SpoVA proteins, as well as cortex hydrolysis by SleB and/or CwlJ. Notably, CaDPA germination of PS4462 spores was more resistant to wet heat than was that of PS4461 spores. This result was surprising, as CwlJ is reported to be only in the cortex/coat fraction of disrupted spores where it might not be wet heat resistant (Bagyan and Setlow 2002, Chirakkal et al. 2002), and how 2Duf's effects on the IM might alter resistance of CwlJ to wet heat is not clear. In another experiment (Fig. 4C), incubation of PS4461 spores at 85°C led to an initial increase in L-valine germination, followed by a large decrease in germination with continued 85°C incubation. In contrast, incubation of PS4462 spores at 85°C for these periods had almost no effect on these spores' L-valine germination (Fig. 4D). However, L-valine germination of PS4462 spores at all heat activation times was slower than that of optimally heat activated PS4461 spores (Fig. 4C and D).

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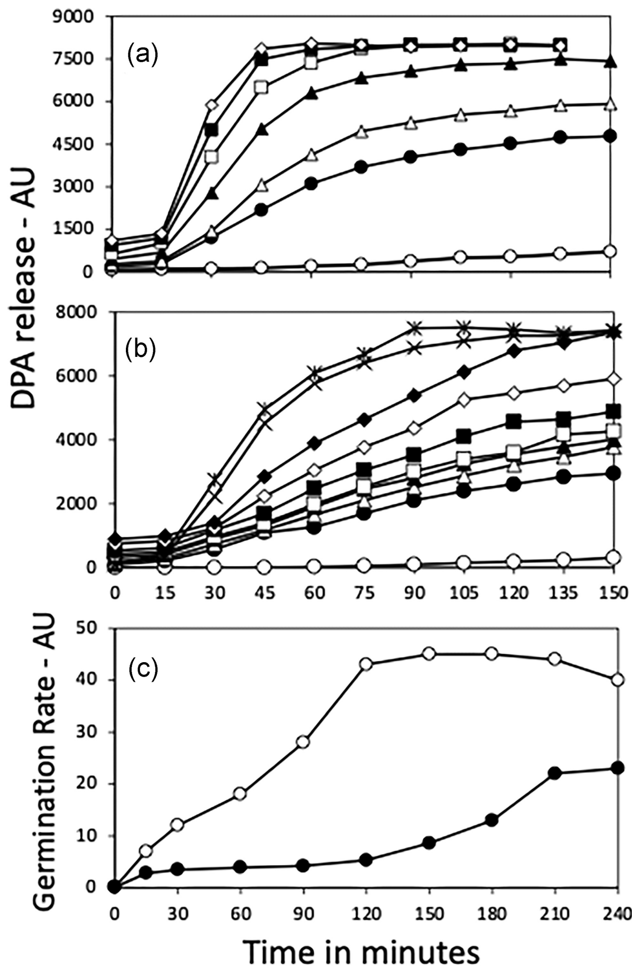

Figure 3. (A–C) Effects of heat activation time on AGFK germination of (A) PS4461 and (B) PS4462 spores. Spores at $\sim 3 \times 10^8$ spores ml^{-1} were incubated at 80°C for various times, and aliquots saved on ice. The spores were germinated at 37°C with AGFK as described in Material and methods section, and CaDPA release monitored fluorometrically in (A and B). Note that amounts of fluorescence at 0 min increased with increasing heating times, more for PS4461 spores than PS4462 spores, presumably due to CaDPA release by the 80°C treatment. (C) Slopes of the initial increases of CaDPA fluorescence for germination, defined as germination rate, after different heat activation times were measured and plotted vs heat activation times. Symbols in A and B denote heat activation times in min as: ○–0; ●–15; △–30; ▲–60; □–90; ■-120; ◇–150; ◆–180; ×- 210; ∗- 240. In (C) the symbols for the rates of germination of the spores are: ○—PS4461; and ●—PS4462.

IM properties in spores +/- 2duf

The results given above indicate that the IM in spores with 2Duf likely has some different properties compared to those of wt spores' IM. To test this directly, we carried out FRAP analyses on spores, as previous analyses showed that a fluorescent probe in spores' IM had relatively low mobility, with a small mobile fraction (Cowan et al. 2004). Thus, we carried out FRAP analysis of a lipid probe incorporated into the IM of PS4461, PS4462, and PS4465 spores, as well as PS533 spores (Cowan et al. 2004). Notably while the small mobile fraction of spores examined was relatively similar, the diffusion coefficient of the lipid probe was ~2-fold lower in PS4462 spores than in PS4461 spores, or in PS4465 spores (Table 2).

Previous work has also shown that spores with 2Duf are more resistant to H_2O_2, nitrous acid, and formaldehyde, three chemicals that kill spores by crossing the IM and damaging either DNA (formaldehyde and nitrous acid) or a crucial core protein (H_2O_2) (Kanaan et al. 2021). Thus, it seems likely that 2Duf in spores will lower IM permeability. To test this, we measured rates and amounts of methylamine uptake into intact or decoated spores (Fig. 5A and B). Intact PS4462 spores had a slightly slower rate of methylamine uptake than intact PS4461 or PS533 spores (Fig. 5A). However, the maximum amount of methylamine uptake was similar in intact spores of all three strains, indicating all three had a core pH of ~6.4 (Table 2). Rates of methylamine uptake by decoated spores were faster than by intact spores (compare Fig. 5B with Fig. 5A), consistent with faster IM water permeability in decoated spores compared to intact spores (Kaieda et al. 2013, Knudsen et al. 2015). Analysis of rates of methylamine uptake in decoated PS4461 and PS4462 spores was complicated by uptake of ~50% less methylamine by PS4462 spores (Fig. 5B), although the PS4462 spores certainly achieved maximum methylamine incorporation faster than the PS4461 spores. The lower maximum uptake of methylamine by PS4462 spores indicates that the pH in decoated PS4462 spores' core is significantly higher than in decoated PS4461 spores' core, but this finding was not studied further.

A question raised by the changes in IM properties in spores with 2Duf is what causes these changes. One possibility is that the IM of spores with 2Duf has an altered phospholipid content, as alterations such as greatly lowered phosphatidylethanolamine (PE) levels significantly decrease spore wet heat and H_2O_2 resistance (Griffiths and Setlow 2009). Consequently, relative levels of various phospholipids were determined in PS4461 and PS4462 growing cells and spores (Table 3). Notably, there were no major differences in IM phospholipid levels in growing cells of these two strains or in spores, although levels of phosphatidylethanolamine (PE) were lower in spores than growing cells, and phosphatidylglycerol (PG) levels and less so cardiolipin (CL) levels were higher in spores. The three major phospholipids, PG, CL, and PE were those found previously in wt spores, although higher percentages of CL were determined in previous work (Griffiths and Setlow 2009). However, one reason for this difference was that in analyzing phospholipid amounts by ^{14}C-acetate labelling in the previous work, that CL contains four fatty acids was not taken into account in phospholipid quantitation.

Discussion

The information in this manuscript on the role of the *spoVA*^{2mob} operon and its *2duf* gene in *B. subtilis* spore wet heat and H_2O_2 resistance strongly suggests that 2Duf exerts its effects by altering properties of the spore IM. Indeed, 2Duf is predicted to be a membrane protein based on its primary sequence and has recently been found in the IM proteome of *Bacillus cereus* spores (Gao et al. 2021; Hao, B., Setlow, P. unpublished results, 2023) but how 2Duf modifies IM structure is not clear. However, the new results, as well as recent work on the resistance of spores +/- 2Duf to these and many other agents (Kanaan et al. 2021), provide significant evidence consistent with a new hypothesis about the mechanism of spore killing by wet heat and H_2O_2, as follows. Previous work (Coleman et al. 2007, Coleman and Setlow 2009, Christie and Setlow 2021) indicated that spore killing by wet heat is: (i) associated with a small amount of spore protein denaturation;

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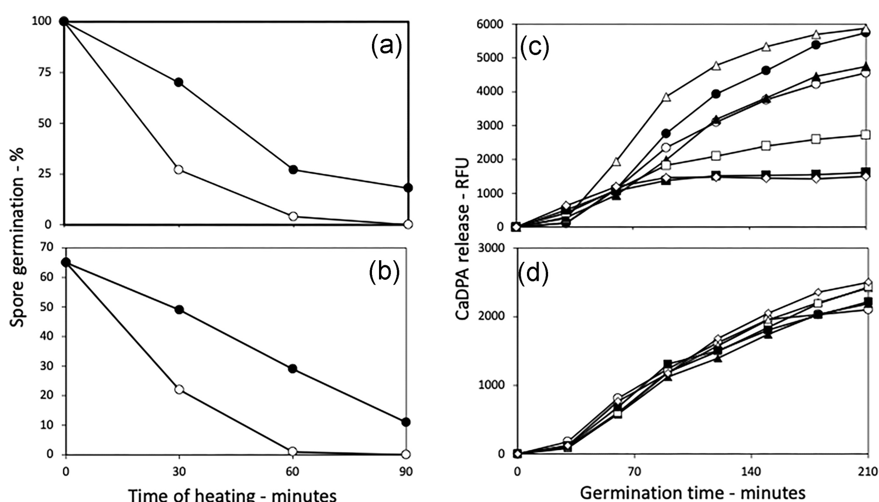

Figure 4. (A–D) Loss of ability to germinate following heat treatment of spores with and without 2Duf. (A) Spores of strains PS4461 (○) and PS4462 (●) at ∼8 × 10^8 ml^{-1} in water were incubated at 93°C (PS4461) or 98°C (PS4462). At various times, two 250 μl aliquots were diluted 4-fold in cold water, centrifuged, the pellets suspended in 500 μl of LB liquid medium with 10 mmol l^{-1} L-valine, incubated at 37°C, and after 90 min, completion of spore germination was measured by phase contrast microscopy of ∼100 spores. (B) Spores of PS4461 (○) and PS4462 (●) at ∼3 × 10^8 ml^{-1} in water were heated at 93°C, aliquots cooled, germinated for 90 min in 40 mmol l^{-1} CaDPA at 23°C and germination assessed by phase contrast microscopy of ∼85 individual spores. In (C) PS4461 and (D) PS4462 spores at ∼3 × 10^8 ml^{-1} in water were incubated at 85°C, aliquots were cooled, and their L-valine germination assessed by measuring CaDPA release in duplicate as described in Material and methods section. The symbols for heating times in min are: ○–0; ●–30; △–60; ▲–90; □–120; ■–150; and ◇–210; note there is no 30 min heating time in (D).

Table 2. *2,4-Di-ANEPPS diffusion coefficients and immobile fractions in the IM of intact spores and the core pH of various intact and decoated spores.

Strain	Diffusion coefficient (μm^2/sec)	Mobile fraction-%	Core pH	
			Intact	Decoated
PS533 (wt)	0.15 +/- 0.04	0.42 +/- 0.10	6.4	6.4
PS4461 (wt)	0.15 +/- 0.06	0.22 +/- 0.08	6.4	6.4
PS4462 (+2Duf)	0.08 +/- 0.03	0.22 +/- 0.11	6.5	7.0
PS4465 (PS4462-2Duf)	0.15 +/- 0.09	0.31 +/- 0.07	Nd†	Nd†

*Spores were labeled with 2,4-Di-ANEPPS and diffusion coefficients and mobile fractions were determined for the dye in the IM of spores of the various strains as described in Material and methods section; note that the dye is lost rapidly from spores more outer layers during spore purification. Spore core pH values were determined from the maximum amount of methylamine incorporated in Fig. 5 (and in data not shown) as compared to that incorporated in PS533 spores, and to the pH in the spore core determined previously in PS533 spores by core methylamine incorporation and measurements with a pH sensitive dye (Swerdlow et al. 1981, Magill et al. 1994).
†Nd—Not done.

Table 3. Relative levels of major phospholipids in growing cells and spores +/- 2Duf.

Samples analyzed	PG	CL	PE	UNK
	Phospholipid levels—% of total +/- SD			
PS4461 vegetative cells	68.1 +/- 10.1	7.0 +/- 0.5	13.6 +/- 0.1	11.3 +/- 1.1
PS4462 vegetative cells	68.6 +/- 2.1	6.8 +/- 0.2	13.4 +/- 0.2	11.3 +/- 0.3
PS4461 spores	77.3 +/- 3.1	11.9 +/- 0.8	6.3 +/- 0.1	4.5 +/- 0.5
PS4462 spores	82.2 +/- 3.6	8.1 +/- 1.2	5.8 +/- 0.02	4.0 +/- 1.7

*Phospholipids were extracted from dormant spores and analyzed by mass spectrometry as described in Material and methods section; values shown are averages from two analyses +/- standard deviations (SD). The abbreviations for the phospholipids are: PG—phosphatidylglycerol; CL—cardiolipin; PE—phosphatidylethanolamine; and UNK—unknown.

and (ii) accompanied, and likely caused by, a loss of the ability to accumulate ATP and other high energy intermediates, such as reduced flavins. Spore killing by H_2O_2 likely damaging a core protein(s) was also accompanied by loss of the capacity of germinated spores to accumulate ATP and reduced FMN (Melly et al. 2002). Notably, other oxidizing agents, including an active chlorine reagent like Sterilox and peroxynitrite (Genest et al. 2002, Loshon et al. 2002, Cortezzo et al. 2004) also do not kill spores by DNA damage, but rather by some unknown damage to spores' IM, and many of the treated germinated spores cannot generate ATP and/or reduced flavins. This previous evidence led to the suggestion that it was damage

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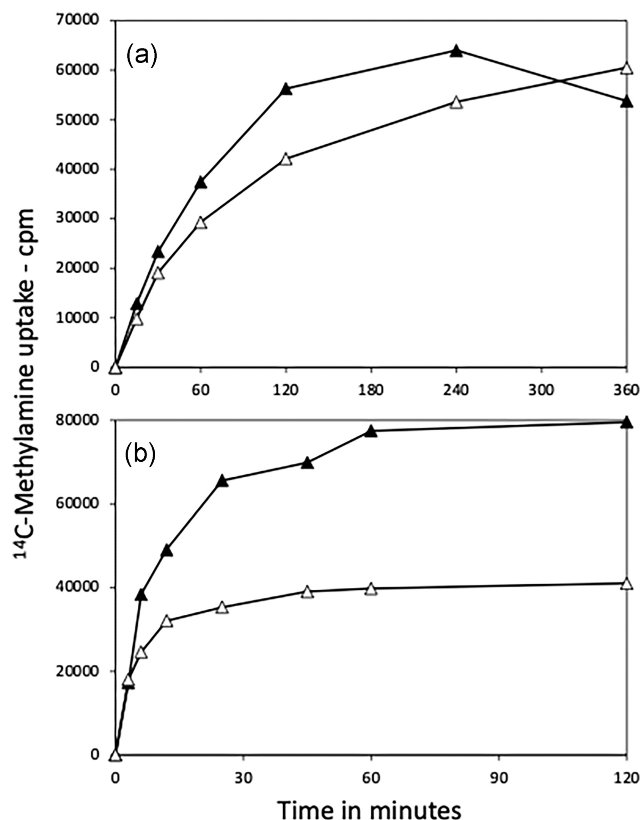

Figure 5. (A and B) ^{14}C-Methylamine uptake by (A) intact and (B) chemically decoated spores. Methylamine uptake was measured as described in Material and methods. All values are averages of duplicate determinations and have been corrected so that uptake into identical numbers of spores is compared. Symbols for spores are: PS4461–▲; and PS4462–△. Values in B are averages of results in two experiments with deviations of ≤15%.

to one or more enzymes of central metabolism that resulted in spore's inability to generate sufficient ATP in LB, whether this be on plates or in liquid, with low ATP levels then leading to spore death. Consistent with the current work, previous findings found that viability of wet heat or H_2O_2-treated spores was increased significantly if glucose was provided in the recovery medium, while Casamino acids alone were much less effective (Coleman and Setlow 2009). In the current work, providing glucose alone with a small amount of L-alanine was almost as effective as 10 mmol l^{-1} total Casamino acids plus glucose in recovery of wet heat or H_2O_2-treated spores. A key conclusion from these findings then is that it is not one or more spore core enzymes of glycolysis that have been damaged by wet heat or H_2O_2, but rather one or more enzymes of oxidative phosphorylation that are essential for: (i) catabolism of amino acids to generate ATP from reduced electron carriers; and (ii) generation of $FMNH_2$ which can be essential for light production by luciferase in bacteria, a process that can be rapidly inactivated by wet heat or oxidizing agent treatment of spores (Genest et al. 2002, Loshon et al. 2002, Melly et al. 2002, Coleman and Setlow 2009).

Invariably, enzymes of oxidative phosphorylation, as well as many enzymes generating and utilizing $FMNH_2$ are in membranes, and a number of these enzymes have been identified in spores' IM (Zheng et al. 2016, Gao et al. 2021). Notably, the *spoVA*^{2mob} operon's encoded 2Duf protein has dramatic effects on spore properties, in particular causing greatly increased resistance of spores with *spoVA*^{2mob} not only to wet heat, but also to three chemicals, H_2O_2, nitrous acid and formaldehyde, which kill spores by damaging either a core protein (H_2O_2) or DNA. (Kanaan et al. 2021). The decreased IM permeability and lower lipid probe mobility due to 2Duf observed in the current work suggests that the IM with 2Duf has a more rigid structure that may stabilize IM proteins against wet heat, and perhaps H_2O_2. Indeed, IM proteins, most notably GRs, and presumably also the SpoVA IM channel proteins for CaDPA, are more resistant or refractory to effects of wet heat in the IM of spores that contain 2Duf than they are in the wt spores' IM. Together these observations are consistent with one or more IM enzymes involved in electron transport and/or oxidative phosphorylation being the key protein(s), damage to which leads to spore death during wet heat treatment and likely with H_2O_2. The challenge now is to obtain direct evidence for this hypothesis, perhaps by looking at denaturation of specific proteins in wet heat-killed spores using proteomic tools that have been developed to analyze heat-denatured proteins in *Escherichia coli* (Leuenberger et al. 2017)

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Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

Conflict of interest

No conflict of interest declared.

Author contributions

George Korza (Investigation, Supervision, Writing—review & editing), Sarah DePratti (Investigation, Writing—review & editing), Daniel Fairchild (Investigation, Writing—review & editing), James Wicander (Investigation), Julia Kanaan (Investigation, Writing—review & editing), Hannah Shames (Investigation, Writing—review & editing), Frank C Nichols (Investigation, Writing—review & editing), Ann Cowan (Supervision, Writing—review & editing), Stanley Brul (Conceptualization, Writing—review & editing), Peter Setlow (Conceptualization, Project administration, Supervision, Writing—original draft, Writing—review & editing).

G.K., S.D., J.K., H.S., D.F., J.W., and F.N. did the experimental work. G.K., A.C., and P.S. supervised the work. S.B. made a crucial suggestion to improve the work. P.S. conceived of the project and wrote the first draft of the manuscript, and all authors edited the manuscript.

Data availability statement

Almost all data are in the manuscript; any reasonable request for additional data will also be accepted.

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