New Thoughts on an Old Topic: Secrets of Bacterial Spore Resistance Slowly Being Revealed

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Summary

The quest for survival is exemplified by spores formed by some Firmicutes. Turning up everywhere one looks, their ubiquity reflects adaptations to the stresses bacteria face, and spores are impactful in public health, food safety and biowarfare. Heat resistance is the hallmark of spores and is countered principally by a mineralized gel-like protoplast/core with reduced water minimizing macromolecular denaturation/aggregation. Dry heat, however, introduces mutations in spore DNA. Spores' countermeasures are multifactorial, including repair in germinated spore outgrowth, but the crystalline-like nucleoid, likely due to DNA saturation with SASP proteins suggests that reduced macromolecular motion is critical in dry heat resistance. SASP are central in spores' radiation resistance, where the contributions of four spore features – SASP, CaDPA, photoproduct lyase, and low water content – minimize DNA damage. Notably, the spore environment steers UV photochemistry towards a product that germinated spores can repair. This resistance extends to chemicals and macromolecules that could damage spores. The latter are excluded by the spore coat which impedes passage of moieties ≥10kDa. Additionally, damaging chemicals may be degraded or neutralized by coat enzymes/proteins. However, principal protective mechanisms here are the inner membrane, a compressed structure lacking lipid fluidity and presenting a barrier to diffusion of chemicals into the spore core; SASP saturation of DNA also protects against genotoxic chemicals. Spores are also resistant to other stresses, including high pressure and abrasion. Regardless, overarching mechanisms associated with resistance seem to revolve around reduced molecular motion, a fine balance between rigidity and flexibility, and perhaps efficient repair.

1.0 Spores – where did they come from?

Many species of Firmicutes, including both aerobic *Bacillales* and anaerobic *Clostridiales*, can cease growth and initiate the process of sporulation, forming endospores from growing cells when environmental conditions have changed so that continued growth is unlikely (1-3). Indeed, a common impetus for initiating sporulation is the depletion of environmental nutrients. However, the decision to form a spore is risky for several reasons. One is that the transformation from a growing cell to a dormant spore is metabolically expensive. In addition, while there is certainly possible gain in acquiring the ability to form spores, as this can mean survival of the population during "bad" times, there are trade-offs in: i) continually maintaining the many genes needed only for sporulation; and ii) ceasing growth, such that the decision to form spores can allow other organisms free rein to grow without competition from spore formers. The upside in this trade-off for the spore formers is of course, that sporulation can lead to survival in bad times due to spores' metabolic dormancy and resistance. Then when good times come and the spores' environment is again conducive to growth, the spores can return to life via germination and then outgrowth (4). That the developmental option of transforming a growing cell into a dormant, resistant spore is indeed advantageous is indicated by the fact that this option evolved more than 2 billion years ago in some anaerobic *Firmicutes* prior to the great oxidation event that allowed the flourishing of the aerobic bacteria, and is still a pathway chosen by many, albeit by no means all present day *Firmicutes* (5, 6). Notably, most aspects of sporulation, spore structure and spore germination are extremely similar in spores of *Bacillales* and *Clostridiales*, and many key spore proteins in the spores of these different species are close homologs (5, 6). Note however, that when sporulation is no longer a useful developmental option, such as when environmental nutrients are constantly replenished, sporulation can be selected against (7, 8), presumably due to

91 the energetic cost of maintaining the many genes needed only for sporulation in the absence of
92 selection.

93 Despite the advantages that spore formation has given to some species of Firmicutes, what is
94 good for spore formers may not be so good for others, including humans, as growing or
95 stationary phase cells of many spore formers are major agents of food spoilage and food
96 poisoning, while some others cause severe human diseases or intoxications including anthrax,
97 botulism, and tetanus, among others (1). Since spores are essentially everywhere in the
98 environment (9), and are most often the vectors of the deleterious effects noted above, there is
99 constant interest in spore eradication, and this is made difficult by spores' high resistance to just
100 about everything (10-13). Indeed, standards for many food sterilization regimens are based on
101 the ability to inactivate spores of particular species of interest, such as toxigenic *Clostridium*
102 *botulinum* (14, 15), and standards for autoclave function, whether steam or chemical, are also
103 often the ability to kill the most resistant spores, such as the very wet heat resistant spores of
104 *Geobacillus stearothermophilus* (16). The mechanism(s) of spore resistance have also been a
105 fertile field for research, with a PubMed search for "bacterial spore resistance" identifying > 650
106 papers over the past 5 years. Notably, surprises can also still appear in this area, such as the
107 relatively recent isolation of spores of the *Bacillus subtilis* group with greatly increased wet heat
108 resistance which appears to involve a novel resistance mechanism (17-19). This review article
109 will re-examine what we do and don't know about spore resistance, focusing on the mechanisms
110 of this resistance as commonly determined in work with spores of the model spore-former,
111 *Bacillus subtilis*. It is certainly hoped that resistance mechanisms elucidated in *B. subtilis* spores
112 will be similar in other spore formers, and this has generally been the case, even with spores of
113 *Clostridiales* (20-22). However, it would be of significant interest and potentially practical value

114 to have more comparative analyses of the mechanisms of the resistance of spores of other species
115 and genera.

116 As noted above, one advantage of spore formation is that because of their dormancy and
117 extreme resistance, spores can survive for long periods in the absence of nutrients and in
118 conditions that might preclude cell survival. Indeed, there have been several reports that claimed
119 or suggested possible spore survival for 10s of millions of years, either entombed in amber or in
120 a liquid inclusion in a crystal in a salt mine (23, 24). In contrast, recent results on projected long-
121 term survival of high concentrations of spores of *B. subtilis* suggest these spores could survive
122 only ~ 10 million years in the presence of natural background γ-radiation (25). However, there
123 are robust data indicating that dry spores of several *Bacillus* species can survive for ≥ 100 years,
124 and then joyously return to life (26). There is also a long-term experiment in progress to monitor
125 spore survival over 500 years (27). For humans, with whom survival for 100 years is generally a
126 stretch, this sounds like spores are doing quite well. Given that there have been billions of years
127 during which these spores have been exposed to all manner of harsh environmental conditions,
128 with continuous selection for spores that can survive, the spores present today are those that have
129 truly stood the test of time. This survival requires that cells of such spore formers undergo two
130 major transitions: 1) from an actively metabolizing cell to a metabolically dormant spore that can
131 survive for many years in the absence of exogenous nutrients, or even metabolism of endogenous
132 nutrients; and 2) from a growing cell sensitive to all manner of severe environmental changes, to
133 a spore resistant to environmental changes such as large fluctuations in temperature, radiation
134 exposure, water availability up to desiccation, and toxic chemicals or enzymes from the
135 environment or other organisms. Since spore forming organisms that were inefficient in these

136 major transitions were at risk of selection against, the spores we see formed today have truly
137 been through the fires of natural selection.
138 There are two major reasons that spores can survive for so long: their dormancy and their
139 resistance. Dormant spores in water exhibit minimal if any metabolic activity, even of
140 endogenous metabolites, and spores of several species accumulate minimal if any ATP even
141 when incubated for long periods at physiological temperatures (28); note that it was imperative
142 that the spores used in the latter referenced work could not complete spore germination, as soon
143 after completion of the latter event, spores rapidly accumulate ATP even if from only
144 endogenous resources. The spore features that generate dormancy appear to be primarily the low
145 water content in the spore core, which can be as low as 25% of wet wt; notably, at least one
146 soluble core protein is immobile in the spore core, yet is fully mobile when spores complete
147 germination and their core water content rises to 80% of wet wt (29). The precise state of core
148 water has been the subject of significant research, with different groups suggesting the spore core
149 is either in a glass like amorphous solid state (30), or a gel like state with significant mobile
150 water, but with spore macromolecules rotationally immobilized (31, 32). The latter model is
151 most consistent with recent detailed studies of spore core water properties by nuclear magnetic
152 resonance techniques (33). The pH in the core, generally ~ 6.5 (34), is also > 1 unit lower than in
153 growing cells and is known to play a crucial role in precluding the activity of a very pH-sensitive
154 spore core enzyme that can initiate catabolism of a major spore energy reserve, 3-
155 phosphoglyceric acid (3PGA), catabolism of which generates ATP soon after completion of
156 spore germination (35). However, 3PGA is not utilized in wild-type dormant *Bacillus* spores that
157 cannot complete germination, due to the absence of both cortex lytic enzymes (CLEs) essential

158 for completion of spore germination, that were incubated for long times at physiological
159 temperatures (28, 36).

160 The second major reason for spores' extreme survival is of course, spores' resistance to
161 all manner of agents (10, 13), including enzymes, wet and dry heat, radiation of multiple types
162 and energy levels, desiccation, high pressures and a host of chemicals and degradative enzymes.
163 Perhaps unsurprisingly, these resistance properties are largely dependent on spores' novel
164 components and structural features.

165

166 ## 2.0 Spore Structural Features and Spore Resistance

167 A major feature responsible for spores' extreme resistance is their novel structure which is very
168 different from that of a growing cell (Fig. 1). The innermost region of the spore is termed the
169 core, and is where spore DNA, RNA and most soluble proteins are located. Given the
170 importance of protecting spore DNA from damage for spores to survive and generate progeny, it
171 is not surprising that some novel spore features help protect spore DNA, and also core proteins
172 essential in macromolecular synthesis and in metabolism. These protective spore features
173 include the low molecular weight small, acid soluble spore proteins (SASP) of the α/β-type that
174 saturate spore DNA (37), the low core water content noted above, and the 1:1 chelate of divalent
175 cations, generally Ca^{2+}, with pyridine-2,6-dicarboxylic acid (CaDPA) that comprises ~ 25% of
176 core dry wt (38). Surrounding the core is the inner membrane (IM), which has a phospholipid
177 and fatty acid composition similar to that of the plasma membrane in growing cells (39).
178 However, the permeability of the IM to small uncharged molecules, including even water, is
179 quite low (32), and lipid probes in the IM are largely immobile as determined by fluorescence
180 redistribution after photobleaching (40). Notably, late in sporulation, ~ 30% of the IM is

extruded into the spore core as the core volume contracts due to its reduced water content (41), most likely under the influence of the spore cortex. However, upon germination when the core volume and then the IM surface area, expands, the IM extruded into the core is assimilated back into the IM.

Just outside the IM there is the first of two layers of peptidoglycan (PG), the germ cell wall which becomes the cell wall of the spore when it completes germination, and the PG structure in this layer appears identical to that in growing cell PG. The second and larger PG layer is the cortex, which has several differences in structure from that of germ cell wall PG, specifically many muramic acid residues are present as muramic acid-δ-lactam and others are attached to only a single alanine residue, instead of a muramyl-peptide with or without a cross-link (42, 43). The novel cortex PG features are recognized by specific hydrolases that initiate cortex-specific PG hydrolysis in spore germination (4, 44). It seems possible that the cortex undergoes expansion late in sporulation, and by pressing against rigid spore coat elements (see below) exerts pressure on the spore core and IM leading to extrusion of core water, forcing some IM material into the core, and decreasing IM permeability (41). Notably, in the absence of most of the spore coat, IM permeability to water increases significantly (45). Outside of the cortex is the outer membrane, which while very important in spore formation, as yet has no known role in spore resistance (10).

The next layer is the spore coat, which itself may have several layers (46). The coat is composed primarily of spore-specific proteins, many of which are cross-linked to give a relatively rigid structure (47, 48), although at least outer layers can flex somewhat (49), and the coat plays some important roles in spore resistance (45, 50-53). The coat is also important in IM permeability as noted above and seems likely to be the rigid layer which ensures that any cortex

204 expansion has effects inward on the IM and core (41). Equally, a recent AFM led study indicated
205 that the cortex is actually more stiff than the coat in *B. subtilis* spores (54). In addition, the coat
206 layer has relatively low permeability to molecules > 10 kDa, something that is important in spore
207 resistance to enzymes that hydrolyze PG (see below) (55). Spores of some species, including *B.*
208 *subtilis*, have an additional relatively thin layer termed the crust just outside of the coat made up
209 of spore specific proteins and glycoproteins (56), but it is not clear if the crust has a specific role
210 in spore resistance (57). Instead of the crust, spores of some other species, for example *Bacillus*
211 *anthracis*, have a seemingly analogous structure, the exosporium, which is similarly made up of
212 spore-specific proteins and glycoproteins (58), but is much more distally located from the coat.
213 While the exosporium may play a role in spore adhesion to abiotic surfaces (59-61), it is not
214 clear if it plays any major roles in spore resistance, although it may prevent large molecules such
215 as antibodies from accessing the spores' more inner layers (55, 62, 63).
216

217 3.0 What are Spores Resistant to – and How Resistant are They?

218 It is often said that spores are extremely resistant, and as noted above, the list of what they are
219 resistant to is long (10), and includes: 1) UV and visible light and ionizing radiation; 2) wet heat,
220 probably the most familiar of spores' resistance properties; 3) dry heat, desiccation and high
221 vacuum; 4) a host of chemicals, including those that alkylate, oxidize or hydrolyze DNA and
222 proteins, including strong oxidizing agents such as peroxides or hypochlorite, as well as
223 aldehydes, acid and bases; 5) enzymes such as lysozyme that degrade cell wall PG, also known
224 as 'eat resistance'; 6) high hydrostatic pressure (HHP), which is used in the food industry; and 7)
225 a number of less well studied agents including high pressure or supercritical gasses, and
226 mechanical disruption.

As is perhaps not surprising, with spores of a particular strain or species, different sporulation media or conditions can give rise to spores with different resistance properties. The resistance property most often studied is to wet heat, but some studies have examined effects of sporulation conditions on spore resistance to dry heat, radiation or chemicals, often hydrogen peroxide (64). Given that spores of several species, notably *Bacillus atrophaeus* and *G. stearothermophilus* are commonly used as biological indicators for autoclave-based sterilization procedures, there have been many studies on effects of sporulation conditions on these spores' wet heat resistance (16, 65, 66). However, effects of sporulation conditions on spores of many other species, including *B. subtilis*, have also been studied (14, 67-71).

Variables in sporulation conditions examined for their effects on spore resistance include: 1) temperature; 2) water activity; 3) pH; 4) divalent cation type and amount; 5) addition of agents increasing the reducing power of sporulation media; and 6) relative medium richness. Much of the results from sporulation with these variables altered are summarized in a relatively recent review on this topic (3). However, while many of these variables can have effects on spore resistance, the reasons for most effects on spore resistance are not known. One exception is sporulation temperature, as within temperatures giving good sporulation, higher temperatures generally give more wet heat resistant spores than lower temperatures. This effect is generally paralleled by lower core water content in spores made at higher temperatures, consistent with the major role for the low core water content in spore wet heat resistance. Another effect of sporulation temperature on spores of *B. subtilis* is some drastic changes in coat assembly in spores made at higher temperatures (71), which might be expected to alter spore wet heat resistance as well as resistance to predation and perhaps some chemicals, but this has not yet been studied in detail.

The addition or removal of specific divalent cations also often has significant effects on spore wet heat resistance (15), and this could certainly alter the divalent cations chelated with DPA, something that can also alter spore wet heat resistance (see below), although the precise explanation for the latter effect is not known. Unfortunately, this type of modulation of sporulation media is generally not followed up by quantitation of levels of divalent cations in the spores produced, and whether these are in the spore core or spores' outer layers, so this is another area where further research is needed.

In addition, the reason(s) for effects of other modifications to sporulation conditions on spore resistance, such as changes in sporulation pH, water activity, reducing power or medium richness are not known, and generally, detailed analyses of the composition of the spores produced in the different conditions have not been carried out. Again, this is an area where further research is needed, in particular to determine the causes of effects of variations in sporulation conditions on spore resistance properties.

Most importantly, examination of the literature indicates that spore resistance to different agents discussed in this article compared to that of growing cells is > 10-fold higher, and most often much, much higher (Table 1). In the sections below, spore resistance to these potential killing agents will be discussed in detail.

3.1 UV and Visible Light and Ionizing Radiation

Spores of Firmicutes are generally 10- to 30-fold more resistant to 254 nm UV radiation, the optimum UV wavelength for damaging DNA, than are growing cells. The major cause of spore UV resistance is the saturation of spore DNA with the α/β-type SASP mentioned above (12). All spore forming *Firmicutes'* genomes have 2-7 genes encoding these very homologous 60-80

273 residue proteins that in total make up ~5% of total spore core protein, and these genes are
274 considered members of the set of genes essential in a spore former (5, 6). The α/β-type SASP are
275 synthesized only in the developing spore core very late in sporulation and are stable in the
276 dormant spore. However, these proteins are rapidly degraded when spore germination is
277 complete, via a process initiated by a SASP-specific endoprotease (72, 73). The free amino acids
278 generated in this degradation provide amino acids for synthesis of most new proteins soon after
279 germination is complete. The α/β-type SASP collectively saturate DNA in spores, and the
280 core's low water content is essential for the saturation, as when spores lacking the SASP-specific
281 protease complete germination and core water content rises to 80% of wet wt, much α/β-type
282 SASP dissociate from the spore chromosome (74). Notably, the three regions of the α/β-type
283 SASP that are the most highly conserved throughout evolution are two that directly bind DNA
284 and the region with the SASP-specific protease recognition sequence (12).
285 Saturation of DNA with α/β-type SASP, either in spores or *in vitro* with purified proteins
286 from a number of species, transitions DNA structure from a B to an A-like conformation (75-77),
287 and this drastically alters DNA's 254 nm UV photochemistry from generation of cyclobutane-
288 type pyrimidine dimers (CPDs) and 6-4-bipyrimidine photoproducts (6-4PPs) in growing cells or
289 DNA *in vitro*, to a thyminyl-thymine adduct termed the spore photoproduct (SP) in spores or
290 DNA saturated *in vitro* with α/β-type SASP from either *Bacillus* or *Clostridium* species (78, 79).
291 Notably, wild type spores of several species are much more UV resistant than spores lacking
292 ~85% of α/β-type SASP, termed $\alpha^-\beta^-$ spores, and UV irradiation of $\alpha^-\beta^-$ spores generates CPDs,
293 and most likely 6-4PPs. The large CaDPA depot in the spore core also is involved in spore UV
294 resistance but sensitizes spore DNA to damage (80).

CPDs and 6-4PPs, as well as SP, can be repaired when spores come back to life and enter outgrowth, and spores contain proteins involved in DNA repair including RecA and SP lyase as well as enzymes of nucleotide excision repair (NER) and base excision repair (BER) (81-83). In addition, a number of genes encoding DNA repair proteins, including RecA, are induced when UV-irradiated spores complete germination, but not in germinated unirradiated spores, such that germinated spores with damaged DNA have higher levels of DNA repair proteins (83). Note that UV treated spores, even if some are killed, can still germinate, something also seen with spores killed by other agents. The CPDs and 6-4PPs are repaired primarily by a cut/patch process involving DNA backbone incision and replication by a low-fidelity DNA polymerase; this repair process is error prone and generates mutations. In contrast, SP repair does not break the DNA backbone as the SP lyase enzyme present in spores monomerizes SP to two thymine residues in an error-free process requiring S-adenosylmethionine once spores have completed germination (84, 85). Since 254 nm UV forms SP and CPD with relatively similar efficiency, it is the error free nature of SP repair compared to that of CPDs and 6-4PPs, which likely explains spore resistance to UV at 254 nm. As expected, spores which lack SP lyase are more sensitive to UV than are wild-type spores and concomitant absence of other DNA repair proteins leads to even lower UV resistance (86). Thus both α/β-type SASP and DNA repair are crucial for full spore resistance to UV at 254 nm. This is also the case for UV at 222 nm, as well as blue light at 400 nm, and SP is again the major photoproduct generated in wild-type spores at these two wavelengths, with minimal CPDs (87, 88).

Coats of spores of some species also contain various pigments, including carotenoids, and there is evidence that such pigments can provide some protection to spores against UV radiation (89, 90), and even more against 400 nm blue light (88). However, these pigments are not the

major source of spore UV resistance, and spores of many species have no such pigments and are still very UV resistant. Spores can also be inactivated by pulsed white light from 200-1100 nm (91, 92), and α/β-type SASP are again important in resistance to this treatment, as is DNA repair (93). The spore coat is also important in resistance to pulsed white light, as spores with severe coat mutations are killed more rapidly than wild-type spores, and this treatment also removes some coat proteins from spores (94). Intriguingly, spores exposed to pulsed near infrared light (700 – 1000 nm) showed only two-fold enhanced resistance to its cidal effects compared to vegetative cells, a surprisingly small deviation in sensitivity, and one that warrants further investigation (95).

As is true for spore resistance to UV and visible radiation, wild-type spore resistance to various types of ionizing radiation is also much greater than that of $\alpha^-\beta^-$ spores (37, 96, 97), indicating that α/β-type SASP binding also protects spore DNA against ionizing radiation. It also seems possible that spores with lower core water contents might exhibit higher ionizing radiation resistance due to less generation of free radicals from water splitting. While there is some evidence consistent with this possibility (96), this topic needs a thorough investigation. As seen with UV radiation, ionizing radiation was found many years ago to generate DNA damage in spores as was shown by identification of increased mutation levels in survivors (98). Consequently, it is not surprising that spore ionizing radiation resistance is increased by the presence of a number of DNA repair enzymes in spores that can act once germination is complete, including enzymes involved in recombination repair, BER repair, abasic site repair and homologous and non-homologous end joining (37, 99, 100). Notably, spore radiation resistance parallels the incorporation of cysteine into developing spores (101), whereas spores produced in cysteine-enriched media show increased resistance to non-ionizing UV between 280

– 400 nm (68). However, the underlying reasons for apparent cysteine-associated resistance to ionizing and non-ionizing radiations have not been established.

3.2 Wet Heat

Resistance to wet heat is the signature resistance property of bacterial spores and given that wet heat treatment is the most commonly used method for spore eradication from foods, guidelines for food sterilization are generally determined by the wet heat temperature and treatment time needed to eradicate the most dangerous spores, such as those of neurotoxin generating *C. botulinum*. Given the widespread use of wet heat for spore killing, it is perhaps not surprising that there has been an enormous amount of research on spore killing by and resistance to wet heat. This work has established that wet heat, including in an autoclave, does not kill spores of several *Bacillus* species by i) DNA damage, or ii) inactivation of one or more essential germination components; or iii) breaking down the spores' IM permeability barrier so that CaDPA is released (16, 102-104); notably, CaDPA release from wet heat-treated spores of several species is well after spore death (102). Importantly, wet heat-treated spore populations, even if killed ~ 95%, can still exhibit percentages of germination > 50% (102, 104). However, only a few percent of these germinated spores enter outgrowth and almost all make minimal if any ATP or other high energy intermediates (104), as has been shown for spores of several *Bacillus* species. This work and other data (105, 106) have led to the suggestion that spore killing by wet heat is due to the inactivation of one or more crucial enzymes in central metabolism in germinated spores – more on this later.

Pioneering work on spore wet heat resistance by Phil Gerhardt and Bob Marquis (107) established a number of additional features of spores that are important in their wet heat

364 resistance as follows: i) the optimum growth temperature of the organism, with spores from
365 organisms with higher optimum growth temperatures and having more intrinsically heat-resistant
366 proteins, being more wet heat resistant, as is the case with spores of the thermophile *Geobacillus*
367 *stearothermophilus*; ii) the degree of mineralization of the spore core and the specific cation(s)
368 associated with DPA, with Ca^{2+} being the cation giving most wet heat-resistant spores and with
369 higher CaDPA levels in the core also generally giving more wet heat resistant spores (note this
370 effect can be difficult to separate from effects of elevated core CaDPA on core water content, as
371 noted below); iii) the sporulation temperature, with higher sporulation temperatures that still
372 allow sporulation giving more wet heat resistant spores; and iv) and most importantly, the water
373 content in the spore core which ranges from 25 to 55% wet wt, in contrast to the usual 80% of
374 wet wt in growing cells, with lower core water contents found in most wet heat resistant spores.
375 Indeed, spores made at higher temperatures generally give spores with lower core water content.
376 One major question about spore core water is how the low spore water content is achieved
377 late in sporulation. A partial answer to this question is that this is due to the developing
378 forespores' uptake of CaDPA made in the mother cell very late in sporulation, with CaDPA
379 uptake paralleled by extrusion of some core water. In *B. subtilis* spores, this reduces core water
380 from 45% of wet wt to ~ 35% of wet wt (108). However, well prior to CaDPA uptake by the
381 developing spore: i) the volume of the spore core has already decreased significantly as has spore
382 core water content (109); and ii) soon thereafter as much as 30% of the developing forespores'
383 IM is extruded into the spore core, and only fuses with the IM when the core expands due to
384 water uptake following CaDPA release and core expansion due to cortex PG hydrolysis in spore
385 germination (41). A major question then is how core volume is decreased in forespore
386 development prior to CaDPA uptake, and the only known candidate for this event is a

mechanical effect of the spore cortex, presumably its expansion against the restraint exerted by the rigid spore coat layer exerting compression inward on the spore core. The importance of the coat in spore wet heat resistance is reflected in the lower wet heat resistance of genetically coatless *B. subtilis* spores (51). In addition, it is now well appreciated that spores undergo what has been termed "maturation" after spores are released from the sporangium (110). This process can lead to large increases in resistance to wet heat and hypochlorite, and is most likely due to increased crosslinking of coat proteins by enzymes in the coat (47, 48), again providing strong evidence for the importance of the coat in spore resistance to wet heat. While the spore cortex expansion against the rigid coat seems a reasonable hypothesis for how much core water is extruded, more work is needed to completely understand this crucial event in sporulation. There is, however, no question that a low core water content can protect proteins against wet heat (108, 111), and studies many years ago indicated that soluble core proteins were stable in wet spores to ~ 40°C higher temperatures than when heated in solution (105).

In addition to the mechanisms noted above, another very important mechanism protecting spores from wet heat is the saturation of spore DNA with the α/β-type SASP described in the section above. At elevated temperatures, both pyrimidine and purine bases in DNA *in vitro* or in growing cells can undergo cleavage of the glycosidic bond generating an abasic site (112), with depurination more rapid than depyrimidination, and both processes are faster at lower pH values (113). Notably, the repair of abasic sites is significantly error prone, and generates mutations, as noted above (113, 114). Since spores of multiple species in water are quite resistant to temperatures >75°C for significant times, and with a spore core with a pH of ~6.5, one would expect there to be much depurination in DNA from heat-treated spores. However, this is not observed, as there are minimal abasic sites in wild-type spores killed 99.9% by wet heat (112). In

410 contrast, wet heat-treated $\alpha^-\beta^-$ spores killed to the same level as the wild-type spores had >20-
411 fold higher levels of abasic sites, and this also correlated with the frequencies of DNA strand
412 breaks in these wet heat- $\alpha^-\beta^-$ treated spores (112). This prevention of depurination of DNA in
413 wild-type spores is almost certainly due to spore DNA's saturation by α/β-type SASP, as: i)
414 these proteins block DNA depurination *in vitro*; and ii) an α/β-type SASP variant that binds
415 DNA poorly does not protect DNA from depurination *in vitro* and does not protect spores well
416 against wet heat (115). DNA binding by α/β-type SASP also greatly inhibits deamination of
417 cytosine residues in DNA (113), another potential mutagenic event. Indeed, the α/β-type SASP
418 protect spore DNA from damage even at elevated temperatures, including at up to > 100°C in a
419 steam autoclave (16). Consequently, spore preparations killed 90-95% by wet heat at various
420 temperatures exhibit no increase in mutation frequency, in contrast to $\alpha^-\beta^-$ spores which: i) are
421 significantly more sensitive to wet heat than are wild-type spores (Table 1); ii) accumulate high
422 levels of mutants in spore populations killed 90-95%; and iii) become even less wet heat resistant
423 if a crucial DNA repair protein such as RecA is also absent (83). In contrast, the loss of RecA
424 has no effect on wild-type spores' resistance to wet heat (83). Presumably, the protection of
425 DNA by α/β-type SASP-binding is so great that spores die from other effects of wet heat well
426 before there is any DNA damage. This other lethal wet heat damage is most likely to spore
427 proteins, and a small amount of protein denaturation takes place during spore killing by wet heat,
428 and prior to CaDPA release, which takes place in only dead, wet heat-treated spores. Following
429 all CaDPA release, there is rapid denaturation of large amounts of spore protein, presumably
430 because of the increased core water content once CaDPA has been released (102). As noted
431 above, there is good evidence that the spore coat also is important in spore resistance, as spores
432 with severely defective coats have significantly lower wet heat resistance. Why the coat is

433 important in wet heat resistance is not completely clear, but it may be multi-factorial i.e., in
434 addition to its rigidity influencing cortex-mediated core volume, it may be because it, along with
435 CaDPA, is important in maintaining a relatively immobile IM environment, as is discussed
436 below.

437 A more subtle effect on spore wet heat resistance is seen when wild-type spores are treated
438 with wet heat applied either conductively or by ohmic (i.e., electrical resistance) heating (116).
439 Notably, ohmic heating kills wild-type spores more rapidly than the same temperature applied to
440 these spores by way of conductive heating. A possible clue to the mechanism of this effect came
441 from demonstrating that $\alpha^-\beta^-$ spores were killed equally well by wet heat applied either
442 conductively or ohmically. These data suggest that spores' high resistance to wet heat due to
443 α/β-type SASP-binding can be subverted by ohmic heat, as the ohmic treatment may weaken
444 interactions between α/β-type SASP and DNA which are not extremely strong. Conceivably,
445 dissociation of the proteins from DNA could lead to heat induced damage on any naked DNA
446 prior to α/β-type SASP-rebinding to DNA when spores are cooled. However, recent work has
447 shown that ohmic heat killing of wt *B. subtilis* spores is not associated with increased
448 mutagenesis (117), so further work is needed to determine exactly how ohmic heat accelerates
449 wild-type spore killing.

450 As a reminder that "nature always finds a way", a new mechanism of spore resistance to
451 wet heat was identified a few years ago in spores of some extremely wet heat resistant spores of
452 *B. subtilis* and its close relatives that had survived in a plant processing foodstuffs at very high
453 temperature (17, 18). The organisms giving these very heat resistant spores had all acquired a
454 transposon with an operon, termed *spoVA*^{2mob}, that was likely expressed only in the developing
455 spore. Notably, these spores' extreme resistance was largely due to a gene on this operon called

456 *2duf*, denoting that it has two domains of unknown function, one being DUF421 (50). These
457 heat resistant spores had neither an elevated CaDPA content nor a decreased core water content
458 (19), indicating that something new was giving rise to the extremely high spore wet heat
459 resistance, and this was likely 2Duf alone or together with some other gene product expressed on
460 the *spoVA*^{2mob} operon; 2Duf seems most likely to be an IM protein, although this has yet to be
461 proven. Notably, spores with 2Duf are highly resistant not only to wet heat, but also to several
462 agents that either modify the IM or must cross it to damage molecules in the core, either proteins
463 damaged by H_2O_2 or DNA damaged by nitrite or formaldehyde (50). It has been known for a
464 number of years that the spore IM has a much lower permeability than the plasma membrane of a
465 growing cell, even to molecules like methylamine or even water (40). This low spore IM
466 permeability is due in part to the spore coat, the removal of which may release cortex pressure on
467 the core such that the IM can expand very slightly, and to the spore core's high level of CaDPA
468 (50). Note that a lipid probe in the spore IM is largely immobile (40), and the IM's lipid
469 mobility and permeability are both increased significantly in spores that lack coats and CaDPA
470 (118); perhaps free coordination sites of Ca^{2+} in CaDPA bind to phospholipid headgroups on the
471 inner IM leaflet and make the IM more rigid and less permeable. While the precise effect of
472 2Duf on IM structure is not well understood, recent results indicate that the presence of the
473 *spoVA*^{2mob} operon makes lipids in the IM more immobile and less permeable to agents that must
474 enter the core to damage core components (50). In addition, the presence of 2Duf stabilizes IM
475 germination proteins to wet heat (119), and makes IM germinant receptors more refractory to the
476 heat activation that can potentiate spore germination (106).
477 The decreased IM permeability of spores containing 2Duf noted above seems likely to be the
478 reason that spores with 2Duf have increased resistance to chemicals that must cross the IM to

cause spore killing (50). While how this effect of 2Duf on IM properties could increase spore wet heat resistance is less obvious, a possible mechanism is suggested when the mode of spore killing by wet heat and hydrogen peroxide is considered, as this appears to be by the inactivation of one or more key enzymes needed for ATP generation in spore outgrowth (102-104, 120). Some of these proteins could be soluble enzymes for glycolysis, as spores have little if any of some enzymes of the tricarboxylic acid cycle (121, 122). However, most spore recovery media often have only low glucose levels and therefore most ATP must be generated in outgrowing aerobic spore formers by oxidative phosphorylation (122), and many of the enzymes for the latter are integral IM proteins (123). Thus, the increased resistance of these enzymes to wet heat and hydrogen peroxide in an IM with 2Duf may be due to a more rigid IM, with 2Duf providing increased resistance to key IM ATP generating proteins. While this is only a hypothesis, it is consistent with several other facts including: i) 2Duf does not provide spores with increased resistance to dry heat or UV radiation, both of which kill spores by DNA damage; and ii) the viability of wet heat-treated spores of several species can be significantly increased by addition of 10 mM glucose to recovery media, but not by addition of substrates such as amino acids that require oxidative phosphorylation to generate energy, and this is also the case for *B. subtilis* spores treated with hydrogen peroxide (103, 124). Clearly further work is needed to fully understand all the effects of 2Duf on spores' IM in detail, and how these effects modulate spore wet heat resistance.

In addition to the effects on spore wet heat resistance noted above, recent work has identified homologs of *2duf* in the *B. subtilis* genome, specifically the *yetF* and *ydfS* genes, as also important in wet heat resistance in *B. subtilis* spores, with a *yetF* deletion reducing wet heat resistance the most (125). Mutations in these two genes also reduced spore resistance to agents

502 that must cross the IM to damage core components, including H_2O_2, nitrite and formaldehyde.
503 Loss of YetF or YdfS also decreased rates of L-valine germination somewhat and AGFK
504 germination even more. The effects of the *yetF* mutation were complemented by re-introduction
505 of the *yetF* gene at the *amyE* locus, and introduction of *yetF* at *amyE* in a wild-type strain
506 increased spore wet heat resistance (125). Importantly, YetF and YdfS are predicted to be
507 membrane proteins and have been found in spores' IM (119). While the CaDPA and core water
508 content of *yetF B. subtilis* spores is identical to those of wild-type spores, the phospholipid
509 composition of spores with and without *yetF* or *ydfS* was also examined, as work 13 years ago
510 showed that changes in specific IM phospholipid levels could decrease *B. subtilis* spore
511 resistance to wet heat and hydrogen peroxide (39). However, analysis of IM phospholipid levels
512 in spores with or without 2Duf, YetF or YdfS found that there were no significant differences
513 between the IM phospholipids in spores of these three strains (120, 125) While these latter
514 results rule out a significant role for IM phospholipids in modulating effects of YetF and its
515 homologs on spore resistance, the initial findings (39) of large effects of changes in IM
516 phospholipid levels on spore wet heat and $H2O_2$ resistance also provide further support for the
517 major role of the IM in determining rates of spore killing by these two agents.
518 The work on effects of YetF and YdfS on spore resistance to date has focused on *B.*
519 *subtilis* spores in which YetF is most likely present in higher levels in spores than in vegetative
520 cells, and perhaps higher than YdfS levels in spores. These two proteins are members of the
521 DUF421 superfamily of proteins that are found in an enormous number of Firmicute spore
522 formers, and which exhibit similar structures when AlphaFold models are compared (125). Even
523 some non-spore formers contain one or more members of this gene family, but to date no
524 function has been ascribed to these proteins in growing cells. Further research into the specific

mechanisms whereby these DUF421 proteins modulate spore resistance seem likely to lead to further surprises.

While most work on spore wet heat treatment has used conductive heating, there are some treatments that generate wet heat by other than conductive means, one such being microwave heating. However, while some work suggested that microwave irradiation of spores of several species had effects greater that a thermal effect alone (126), others have found no such additive effect (127-130), and there has been no significant study of the mechanisms of spore resistance to microwave heating. There are also treatments that combine a thermal effect with another treatment; one such is carrying out sonication of spores at an elevated temperature, a process termed thermosonication (TS) (131-135). TS kills spores of many species, and with *B. subtilis* spores, the factors important in resistance to this treatment are most importantly the α/β-type SASP (134), although evidently TS-treatment results in damage to the inner membrane and spore coat (132, 134, 135).

3.3 Dry Heat, Desiccation and High Vacuum

Spores are generally resistant to ~ 20°C higher temperatures when dry than when heated in water, but can be killed by dry heat, and this treatment is used in some situations requiring sterilization, including in spacecraft assembly (136). As with wet heat, the α/β-type SASP are also an important factor in spore dry heat resistance, as DNA damage by dry heat, including depurination, is greatly slowed by DNA's saturation with α/β-type SASP, and $\alpha^-\beta^-$ spores are more sensitive to dry heat that wild-type spores (114). However, unlike with wet heat, wild-type spore populations killed 90-95% by dry heat accumulate high levels of mutations (114, 137, 138). Consequently, DNA repair when spores begin outgrowth is also important in their dry heat

548 resistance and mutations in major DNA repair proteins such as RecA greatly decrease spore dry
549 heat resistance (83). The DNA damage generated by dry heat treatment of spores includes both
550 transitions and transversions (139).
551 Wild-type spores are generally very resistant to desiccation alone and can survive multiple
552 cycles of hydration and desiccation at moderate vacuum (~ 10 Pa) (140). The α/β-type SASP are
553 a major factor in spore desiccation resistance at moderate vacuum, as $\alpha^-\beta^-$ spores are killed 30-
554 75% by even one cycle of desiccation under moderate vacuum and then rehydration, with
555 multiple cycles giving more and more killing; it is the processes of either desiccation or
556 rehydration or both that give the killing of the $\alpha^-\beta^-$ spores, not the time in the desiccated state
557 (140). Again, DNA damage seems to be the mechanism of the killing of the $\alpha^-\beta^-$ spores by
558 desiccation, as there is increased mutagenesis of the survivors and the presence of DNA repair
559 enzymes in spores is important in $\alpha^-\beta^-$ spores' desiccation resistance. While wild-type spores
560 survive desiccation at moderate vacuum, desiccation at high vacuum ($\leq$ 0.1 Pa) results in these
561 spores' killing, and by DNA damage (27, 141). Again, the α/β-type SASP as well as DNA
562 repair in spore outgrowth are most important in resisting spore killing by high vacuum (99, 139,
563 142, 143).
564

565 ## 3.4 Chemicals

566 Spores can be killed by a wide range of chemicals including a variety of DNA damaging agents,
567 some amines and alcohols, and oxidizing agents, aldehydes, acids, and bases (11, 144). For
568 potential DNA damaging chemicals that can alkylate, deaminate or oxidize DNA, spore
569 resistance to these agents is due to: 1) the low IM permeability, as increases or decreases in IM
570 permeability correlate with more rapid or slower spore killing, respectively (120, 124, 145); 2)

571 DNA protection by α/β-type SASP, which is so effective that several peroxides including
572 hydrogen peroxide do not kill wild-type spores by DNA damage, but most likely by oxidative
573 damage to amino acid residues, in particular methionines, in one or more core enzymes (146-
574 148). For example, even α/β-type SASP bound to DNA in spores can undergo oxidation of
575 methionine residues by multiple peroxides, as can methionines in other core proteins, and these
576 agents can also oxidize lysine γ-amino groups in core proteins to aldehydes (149). Presumably
577 then it is by protein damage that wild-type spores are killed by these peroxides. However, the
578 specific protein or proteins oxidative damage to which leads to spore death is not known,
579 although killed peroxide-treated spores can germinate, albeit slowly (150). Indeed, germinated
580 hydrogen peroxide-killed spores do not accumulate ATP, as is the case with germinated wet
581 heat-killed spores, and perhaps both agents target similar IM enzymes involved in oxidative
582 phosphorylation (102, 120, 151). In contrast to the behavior of wild-type spores, at least
583 hydrogen peroxide kills $\alpha^-\beta^-$ spores by DNA damage, and DNA repair in peroxide-treated
584 outgrowing $\alpha^-\beta^-$ spores is important in these spores' resistance (83).
585 Larger, more hydrophilic potential DNA damaging agents, especially if charged at
586 neutral pH, do not kill spores by DNA damage. This group of chemicals includes strong
587 oxidizing agents such as hypochlorite, chlorine dioxide or ozone, which do not kill spores by
588 DNA damage as they presumably do not cross the IM, do not kill $\alpha^-\beta^-$ spores more rapidly than
589 wild-type spores, and loss of DNA repair capacity has no effect on spore resistance to such
590 agents (152, 153). In addition to prevention of damage to spore core components by low IM
591 permeability, major protection against these oxidizing agents as well as aldehydes such as
592 glutaraldehyde is provided by the spore coat, as severe coat defects result in more rapid spore
593 killing by such agents. However, decoated wild-type spores treated with such agents still are not

killed by DNA damage, and even decoated $\alpha^-\beta^-$ spores are not killed by DNA damage (152-155). Thus, the major protection against such agents is likely by their detoxification in reactions with spore coat protein, so there is less damage to more inner spore layers. Indeed, the lethal damage whereby such agents kill spores appears to be in the IM, as while the killed spores can germinate, the IM often ruptures when the core swells in germination. However, the specific IM damage that causes this effect is not known but is not oxidation of unsaturated fatty acids (124).

Another potential protective feature of the coat of spores of some species is that potential detoxification enzymes such as catalase and superoxide dismutase are present not only in the spore core but also in the coat (156, 157). The core enzymes play no role in spore resistance (158), even if their levels are greatly increased (159), presumably because these enzymes are inactive in the core's low water content, but they can increase the resistance of the fully germinated spore, in which they become active (160). There is also evidence that at least superoxide dismutase in the coat of *Bacillus anthracis* spores can increase spore resistance to agents that generate superoxide (161).

Spores are also resistant to low and high pH values, such as with 1 M acid or base, but pH extremes do not kill spores by DNA damage, and loss of DNA repair, α/β-type SASP or spore coats do not affect spore resistance to these pH extremes, which is not really understood. However, findings with spore resistance to strong alkali has exemplified some of the potential for misleading results that can arise in studies of spore killing by chemicals, as while *B. subtilis* spores treated with 1 M NaOH may give no colonies on nutrient plates, colony formation is fully restored on plates with low levels of lysozyme (162). Notably, given germinants, these alkali-treated spores rapidly release CaDPA in the initial step in spore germination, but cannot degrade the cortex PG and complete the germination process (150). Presumably, the cortex-lytic enzymes

617 are inactivated by strong alkali, and lysozyme was a surrogate for these enzymes, as the treated
618 spores were not truly dead. Another more insidious problem in assessing spore killing by
619 chemical agents is that unless these are removed or fully inactivated, when spores are plated on
620 nutrients to allow spore germination, toxic chemicals that have been carried over can rapidly kill
621 the much more sensitive germinated spores. Sadly, this problem has led to more than one error in
622 classifying a chemical agent as being able to kill dormant spores (163, 164).
623

624 ## 3.5 Eat resistance

625 Sporulating cells spend a large amount of time and energy in making all the specific proteins that
626 comprise the spore coat, $\geq$ 80 proteins in *B. subtilis*, which together make up >50% of total spore
627 protein. Given this large energy expenditure, one would presume that the coat provides some
628 major benefit to spores, and indeed it does, as it deals with a major problem spores face when in
629 soil or water which have many other types of organisms, including lower eukaryotes and
630 predatory bacteria, for all of whom bacteria are their favorite meal. The eukaryotic bacterivores
631 ingest "meals" of soil or water, and enzymes in their digestive tract, most importantly PG
632 hydrolases that lyse and kill any ingested bacteria, together with other hydrolases digest the
633 "meal" to metabolites, allowing the predator to flourish. However, in this predator-rich
634 environment, the spore can neither flee from its predators nor release some chemical defense but
635 must stand its ground. This is where the spore coat comes in, as several studies have shown that
636 intact spores of the model organism *B. subtilis*, a soil organism, are readily taken up either by the
637 protozoan *Tetrahymena thermophilus* or the nematode *Caenorhabditis elegans*. However, the
638 ingested spores are neither killed nor digested, but rather are excreted no worse the wear from
639 their brief sojourn in these predator's digestive tract (52, 53). Notably, the spores' resistance to

these predators is dependent on their coat, as severely coatless spores are readily eaten, with increased sensitivity to digestion correlating with more severe coat defects. There are also multiple reports of spores of many different species surviving transit through the human gut (165, 166), although there have been no studies on the fate of coatless spores in the human gut.

There are also at least two species of bacteria, *Myxococcus xanthus* and *Cupriavides necator*, that prey on other bacteria, including growing *B. subtilis* cells, but cannot kill their spores (167). However, the mechanism of the resistance of spores to these predatory microbes is either not known (*M. xanthus*) or does not involve spore coats (*C. necator*) and may be due to spores' dormancy and low IM permeability. Note that spores' dormancy and low IM permeability also preclude spore killing by antibiotics.

3.6 High Hydrostatic Pressure (HHP)

Spores are markedly resistant to HHP, perhaps because of their relatively rigid layers, but HHP resistance does not involve an intact spore coat (168). However, HHP treatment can potentiate spore killing by triggering their germination at either moderate pressures (150-330 MPa) or much higher pressures (up to 900 MPa) (169, 170). While germinated spores can be killed by HHP, generally this is not efficient, and in the food industry HHP treatment is carried out at high temperatures to kill the partially or completely germinated spores, which are much less wet heat resistant that are dormant spores (135, 171-174).

3.7 Mechanical Disruption

Spores are much more resistant to mechanical disruption than growing cells, whether by sound waves (sonication) (131-135), by shaking with glass beads (175) or piercing by silicon

663 nanopillars (176). However, while studies of this type of spore killing have ruled out specific
664 protective components such as α/β-type SASP and most spore core proteins, the specific
665 mechanisms involved in spore resistance to mechanical disruption have not been identified, and
666 even removal of much of the spore coat does not sensitize spores to mechanical disruption or
667 piercing (175, 176).
668

669 ## 3.8 Miscellaneous

670 The past decade or so has seen the development and introduction of a fairly wide range of
671 technologies aimed at inactivating spores in environments where traditional thermal and
672 chemical approaches may not be appropriate (177). Various non-thermal gas plasma-based
673 approaches have received considerable attention (178, 179), although knowledge of spore
674 components damaged by non-thermal and low pressure plasmas and resistance counter measures
675 is incomplete. However, exposure of spores to cold atmospheric plasma was reported to damage
676 the spore inner membrane and germination proteins, with the damage being caused by charged
677 particles and reactive oxygen species as opposed to UV radiation (180). Configuration of plasma
678 devices seem likely to modulate spore targets since a low temperature nitrogen-oxygen plasma
679 system was found to damage spore DNA via emission of UV-C, and that SASP and DNA repair
680 enzymes conferred resistance in this regard (181). Another emerging approach concerns the
681 application of supercritical gasses, usually CO_2 coupled with chemical modifiers for sterilizing
682 heat sensitive surfaces in the healthcare and food sectors (182, 183). Few studies have pursued
683 the physiological basis for spore damage in this regard, however, spores exposed to supercritical
684 CO_2 with peracetic acid were killed principally by damage to the inner membrane (184), with the
685 coat and SASP proteins conferring varying degrees of resistance to wet and dry spores

686 respectively. Finally, at the opposite end of the thermal spectrum, exposure of spores to blast
687 environment conditions, that is ~1000^{o}C for 10s of milliseconds, resulted in efficient spore
688 killing via damage to unknown spore proteins and perhaps the inner membrane (185). Evidently
689 there are limits to resistance, even for these most recalcitrant of microorganisms.
690

691 ## 4.0 Concluding Remarks

692 After more than seven decades of intensive research driven in no small part by the quest to
693 eradicate, spores continue to confound and surprise. Just when progress in understanding the
694 basis of resistance might have been in danger of plateauing new players (proteins (17-19, 125))
695 suddenly emerge, adding to the mosaic of features that collectively underpin spore recalcitrance.
696 How were these missed for so long in a busy field of research? If anything, recent events in the
697 field demonstrate once again that spores do not easily give up their secrets. Indeed, an
698 assessment of the literature, such as in this review, suggests that current knowledge of spore
699 resistance to a number of important stress factors frequently lacks granularity. Hence, as we
700 move forward the objective should be adding detail to 'the damaged inner membrane', or the
701 'undefined core protein', or more precisely defining what is meant by 'coat disruption'. Which
702 proteins or lipids? What is the effect at the nano and ultrastructural levels? What new and
703 emerging techniques can we apply to probe the spore in such detail, and can we then use this
704 knowledge to more efficiently kill spores? Clearly there is much left to be learned about spores,
705 their resistance, and their ever elusive Achilles' heel.
706
707
708

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Table 1 Resistance of growing wild-type *Bacillus subtilis* cells, wild-type spores, and wild-type spores carrying the *spoVA*^{2mob} transposable element to various treatments

Treatment	Growing cells (wild-type)[d]	Dormant spores (wild-type)[e]	Dormant spores (*spoVA*^{2mob})[e]	Key resistance factors
Wet heat (93°C)[a]	<0.05	35	>120	Low core water, SASPs, transposon gene(s), Coat
Dry heat (120°C)[a]	<5	170	200	SASPs
UV at 254 nm[a]	<0.1	1.2	1.3	SASPs, DNA repair, Coat
NaOCl (2.5% available chlorine)[a]	<0.1	4.5	6.5	Coat
H_2O_2 (11%)[a]	<0.5	205	280	SASPs, Coat, IM
HCHO (2.5%)[a]	<0.1	20	>60	SASPs, Coat, IM

HNO_2 (200 mM)[a]	<0.1	15	>40	SASPs, IM
$C_{12}H_{27}N$ (1 mM)[a]	ND	20	>60	IM
Desiccation[b,c]	<1	>20	ND[f]	SASPs
γ-radiation (100 krads)[c]	1	28	ND[f]	SASPs, DNA repair

[a] Time in minutes to kill 90% of the population

[b] number of freeze thaw cycles

[c] % survivors

[d] data from (12) except where indicated

[e] data from (50) except where indicated

[f] ND, not determined

Figure 1

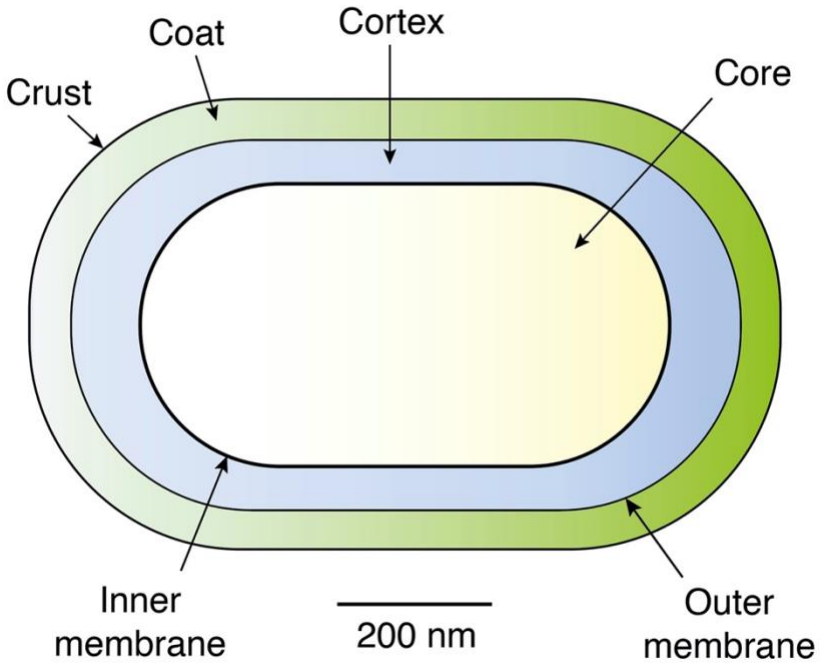

Figure 1 Schematic of a longitudinal cross-sectioned *Bacillus subtilis* spore showing the major morphological features visible by transmission electron microscopy. The germ cell wall is not distinguished but is located between the cortex and inner membrane. Spores of some species have an additional thin layer of coat-like material, the exosporium, which unlike the *B. subtilis* crust, is usually located distally with respect to the coat.

Figure 2

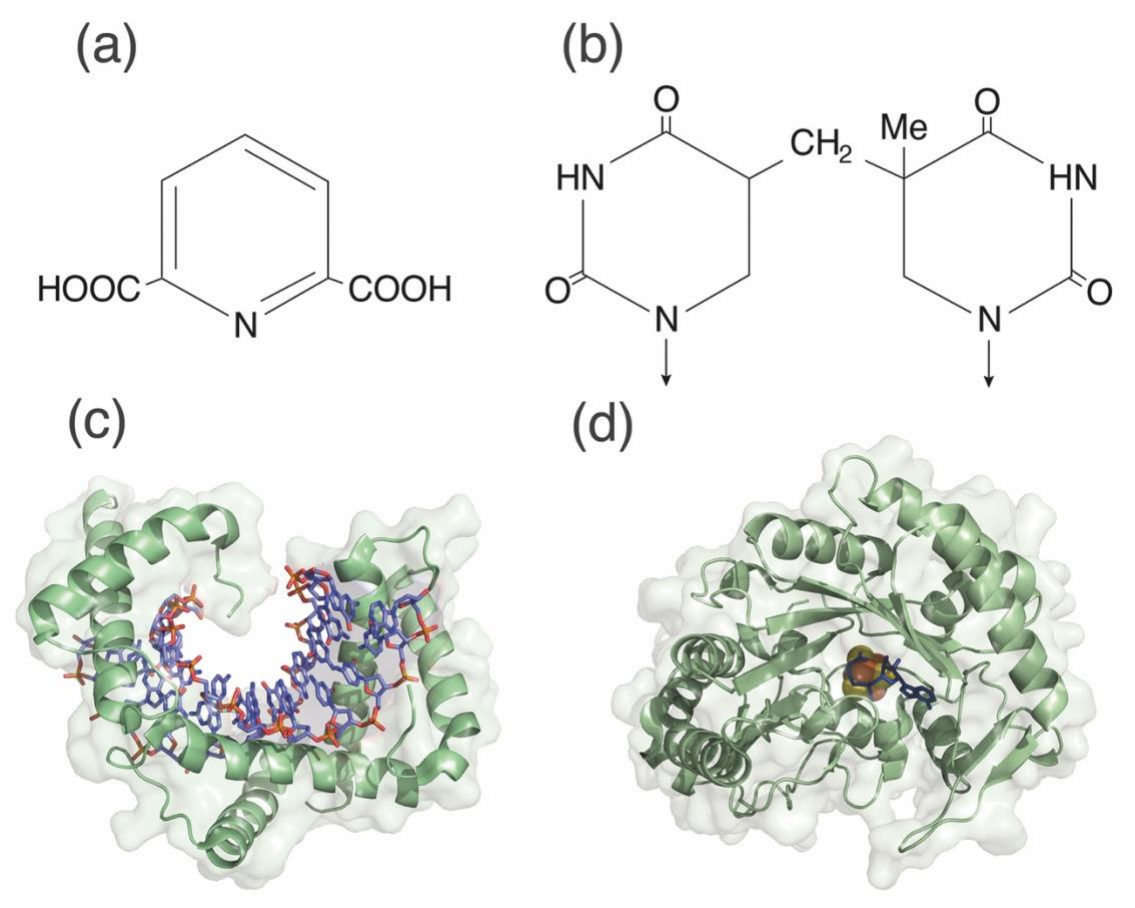

Figure 2 A quartet of spore resistance-associated molecules. (a) pyridine-2,6 -dicarboxylic acid (DPA), which would be charged and chelated with Ca^{2+} ions in the spore core; (b) thyminyl-thymine adduct, often referred to as spore photoproduct (SP); (c) SASP – DNA complex (DNA, blue & orange) [PDB:2z3x]; (d) SP lyase with S-adenosyl-L-methionine cofactor (blue) bound at the iron-sulfur cluster (yellow and orange) [PDB:4fhc].