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REVIEW

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

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**SUMMARY** The quest for bacterial survival is exemplified by spores formed by some *Firmicutes* members. They turn up everywhere one looks, and their ubiquity reflects adaptations to the stresses bacteria face. 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, termed the spore core, with reduced water which minimizes macromolecular movement/denaturation/aggregation. Dry heat, however, introduces mutations into spore DNA. Spores have countermeasures to extreme conditions that are multifactorial, but the fact that spore DNA is in a crystalline-like nucleoid in the spore core, likely due to DNA saturation with small acid-soluble spore proteins (SASPs), suggests that reduced macromolecular motion is also critical in spore dry heat resistance. SASPs are also central in the radiation resistance characteristic of spores, where the contributions of four spore features—SASP; $Ca^{2+}$, with pyridine-2,6-dicarboxylic acid (CaDPA); photoproduct lyase; and low water content—minimize DNA damage. Notably, the spore environment steers UV photochemistry toward a product that germinated spores can repair without significant mutagenesis. This resistance extends to chemicals and macromolecules that could damage spores. Macromolecules are excluded by the spore coat which impedes the passage of moieties of $\geq$10 kDa. Additionally, damaging chemicals may be degraded or neutralized by coat enzymes/proteins. However, the principal protective mechanism here is the inner membrane, a compressed structure lacking lipid fluidity and presenting a barrier to the 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.

**KEYWORDS** bacterial spores, spore resistance



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## INTRODUCTION

Many species of *Firmicutes*, including those in both aerobic *Bacillales* and anaerobic *Clostridiales* groups, 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 reason is that the transformation from a growing cell to a dormant spore is metabolically expensive, i.e., many vegetative cell proteins initially in the developing spore will be degraded, many spore-specific proteins are made, and the mother cell dies, and its degradation products are then free to anyone else in the environment (1), all with no increase in cell numbers. In addition, while there is certainly a possible gain in acquiring the ability to form spores, as it can mean the 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 the metabolic dormancy and resistance properties of spores. Then, when the spore 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* members prior to the great oxidation event that allowed the flourishing of the aerobic bacteria, and it is still a pathway chosen by many *Firmicutes* species (5, 6). Notably, most aspects of sporulation, spore structure, and spore germination are similar in spores of *Bacillales* and *Clostridiales*, and many key spore proteins 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), perhaps (i) due to the energetic cost of maintaining the many genes needed only for sporulation and (ii) in the absence of selection for the function of these genes.

Despite the advantages that spore formation has given to some *Firmicutes* species, what is good for spore formers may not be beneficial for other organisms, including humans, as growing or stationary-phase cells of many spore formers are major agents of food spoilage and food poisoning, while some others cause severe human diseases or intoxications, including anthrax, botulism, and tetanus, among others (1). Since spores are essentially everywhere in the environment (9) and are most often the vectors of the deleterious effects noted above, there is constant interest in spore eradication, which is made difficult by the high resistance of spores to just about everything (10–13). Indeed, standards for many food-sterilization regimens are based on the ability to inactivate spores of particular species of interest, such as toxigenic *Clostridium botulinum* (14, 15). Standards for autoclave function, whether steam or chemical, are often the ability to kill the most resistant spores, such as the very wet heat resistant spores of *Geobacillus stearothermophilus* (16). The mechanism(s) of spore resistance have also been a fertile field for research. A contemporary PubMed search for "bacterial spore resistance" identifies >650 papers over the past 5 years. Notably, advances still appear in this area, such as the relatively recent isolation of spores of the *Bacillus subtilis* group with greatly increased wet heat resistance, which appears to involve a novel resistance mechanism (17–19). This review article will reexamine what we do and do not know about spore resistance, focusing on the mechanisms of this resistance as commonly determined in work with spores of the model spore former *Bacillus subtilis*. Hopefully, resistance mechanisms elucidated in *B. subtilis* spores will be similar to those in other spore formers, and this similarity has generally been the case, even with spores of *Clostridiales* members (20–22). However, it would be of significant interest

and potentially practical value to have more comparative analyses of the mechanisms of the resistance of spores of other species and genera.

As noted above, one advantage of spore formation is that because of their dormancy and extreme resistance properties, spores can survive for long periods in the absence of nutrients and under conditions that might preclude cell survival. There have been several controversial reports that claimed or suggested possible spore survival for tens of millions of years, either entombed in amber or in a liquid inclusion in a crystal in a salt mine (23, 24), but it is certainly not clear if these claims are valid. Indeed, very recent results on the projected long-term survival of spores of *B. subtilis* suggest that even with high concentrations of these spores, one individual could survive at most only ~10 million years when dry and exposed to natural background gamma radiation (25). However, there is one well documented report indicating that dry spores of several *Bacillus* species can survive for ≥100 years and then joyously return to life (26). There is also a long-term experiment in progress to monitor spore survival over 500 years (27). For humans, with whom survival for 100 years is generally a stretch, this sounds like spores are doing quite well. Given that there have been billions of years during which spores have been exposed to all manner of harsh environmental conditions, with the continuous selection for spores that can survive, spores present today are those whose ancestors have truly stood the test of time. This survival requires that cells of such spore formers undergo the following two major transitions: (i) from an actively metabolizing cell to a metabolically dormant spore that can survive for many years in the absence of exogenous nutrients or even the metabolism of endogenous nutrients and (ii) from a growing cell sensitive to all manner of severe environmental changes to a spore resistant to environmental changes, such as large fluctuations in temperature, radiation exposure, water availability up to desiccation, and toxic chemicals or enzymes found in the environment or produced by other organisms. Since spore-forming organisms that were inefficient in these major transitions were at risk of being selected out, the ancestors of the spores we see formed today have truly been through the fires of natural selection. Equally, it is worth bearing in mind the distinction between resistance properties selected for over evolutionary timescales and which pertain to physiological and or ecological processes versus those that are more likely to be fortuitous and are merely incidental to the original stress factor. The former properties include resilience in the face of predation, desiccation, and UV radiation, whereas the latter properties are generally resistance properties against anthropologic-associated stressors, such as extremes of heat (at least for spores of mesophiles), mechanical disruption, and exposure to organic solvents and various other toxic (often manufactured) chemicals.

There are two major reasons that spores can survive for so long, namely, their dormancy and their resistance. Dormant spores in water exhibit minimal, if any, metabolic activity, even of endogenous metabolites, and spores of several species accumulate minimal if any ATP even when incubated for long periods at physiological temperatures (28). Note that it was imperative that the spores used in reference 28 could not complete germination, as soon after this event, spores rapidly accumulated ATP even if from only endogenous resources. As a consequence of their dormancy, spores (i) are not at risk from starving to death and (ii) will produce minimal, if any, toxic by-products of oxidative metabolism that can damage DNA and proteins. The spore features that generate dormancy appear to be primarily the low water content in the spore core, which can be as low as 25% of wet weight; notably, at least one soluble protein in the spore core, the reporter fluorophore green fluorescent protein (GFP), is immobile in the spore core and yet is fully mobile when spores complete germination and their core water content rises to 80% of wet weight (29). The precise state of core water has been the subject of significant research, with different groups suggesting the spore core is in either a glass-like amorphous solid state (30) or a gel-like state with significant mobile water but with spore macromolecules rotationally immobilized (31, 32). The latter model is most consistent with recent detailed studies of spore core water properties by nuclear magnetic resonance techniques (33). The pH in the core, generally ~6.5 (34), is >1 unit lower than that in growing cells, which plays a crucial role in

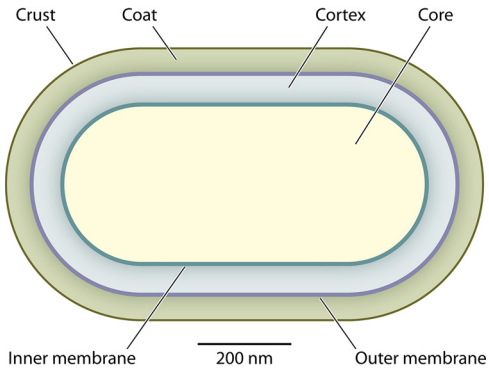


**FIG 1** Schematic of a longitudinal cross-sectioned *Bacillus subtilis* spore showing the major morphological features visible by transmission electron microscopy. The germ cell wall is not distinguished but is located between the cortex and inner membrane. Spores of some species have an additional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

precluding the activity of a very pH-sensitive spore core enzyme that can initiate the catabolism of a major spore energy reserve, 3-phosphoglyceric acid (3PGA), of which its catabolism generates ATP soon after the completion of spore germination (35). Indeed, the ATP generated from 3PGA is essential for maintaining the viability of spores germinating in a poor medium (36). However, 3PGA is not utilized in mutant *Bacillus* spores that cannot complete germination because of their lack of both cortex lytic enzymes (CLEs) essential for the completion of spore germination, even upon incubation with germinants for long times at physiological temperatures (28, 37).

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

## SPORE STRUCTURAL FEATURES AND SPORE RESISTANCE

A major feature responsible for the extreme resistance of spores is their novel structure which is very different from that of a growing cell (Fig. 1). The innermost region of the spore is termed the core and is where spore DNA, RNA, and most soluble proteins are located. Given the importance of protecting spore DNA from damage for spores to survive, it is not surprising that some novel spore features help protect spore DNA (Fig. 2). Moreover, spores also have strategies to protect core proteins essential in macromolecular synthesis and metabolism. These protective spore features include the low-molecular-weight small acid-soluble spore proteins (SASPs) of the $\alpha/\beta$ type that saturate spore DNA (38), the low core water content noted above, and the 1:1 chelate of divalent cations, generally $Ca^{2+}$, with pyridine-2,6-dicarboxylic acid (CaDPA) (Fig. 2A) that comprises ~25% of core dry weight (39). Surrounding the core is the inner membrane (IM), which has a phospholipid and fatty acid composition similar to that of the plasma membrane in growing cells (40). However, the permeability of the IM to small uncharged molecules, including even water, is quite low (32), and lipid probes in the IM are largely immobile as determined by fluorescence redistribution after photobleaching (41). Notably, occurring late in sporulation, ~30% of the IM is extruded into the spore core as the core volume contracts due to its reduced water content (42). However, upon germination when the core volume and the IM surface area expand, 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 (1). 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-$\delta$-lactam and

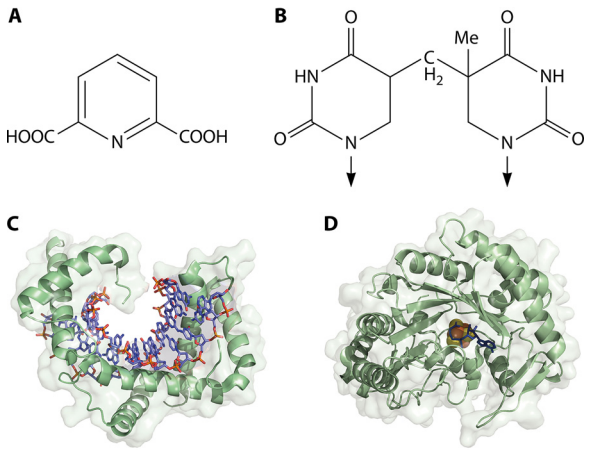


**FIG 2** A quartet of spore resistance-associated molecules. (A) Pyridine-2,6-dicarboxylic acid (DPA), which would be charged and chelated with $Ca^{2+}$ ions in the spore core; (B) thyminyl-thymine adduct, often referred to as spore photoproduct (SP); (C) SASP-DNA complex (DNA, blue and orange) (Protein Data Bank [PDB] 2z3x); (D) SP lyase with *S*-adenosyl-L-methionine cofactor (blue) bound at the iron-sulfur cluster (yellow and orange) (PDB 4fhc).

others are attached to only a single alanine residue, instead of a muramyl-peptide with or without a cross-link (43, 44). The novel cortex PG features are recognized by specific hydrolases that initiate cortex-specific PG hydrolysis in spore germination (4, 45). 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 (42). Notably, in the absence of most of the spore coat, IM permeability to water increases significantly (46). 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 (47). The coat is composed primarily of spore-specific proteins, of which many are cross-linked to give a relatively rigid structure (48, 49), although at least outer layers can flex somewhat (50), and the coat plays some important roles in spore resistance (46, 51–54). The coat is also important in IM permeability as noted above and seems likely to be the rigid layer which ensures that any cortex expansion has effects inward on the IM and core (42). Equally, a recent atomic force microscope (AFM) led study indicated that the cortex is actually more stiff than the coat in *B. subtilis* spores (55). In addition, the coat layer has relatively low permeability to molecules (>10 kDa), which is something that is important for spore resistance to enzymes that hydrolyze PG (see below) (56). Spores of some species, including *B. subtilis*, have an additional relatively thin layer termed the crust just outside the coat made up of spore-specific proteins and glycoproteins (57), but it is not clear if the crust has a specific role in spore resistance (58). Instead of the crust, spores of some other species, for example *Bacillus anthracis*, have a seemingly analogous structure, the exosporium, which is similarly made up of spore-specific proteins and glycoproteins (59) but is much more distally located from the coat. While the exosporium may play a role in spore adhesion to abiotic surfaces (60–62), it is not clear if it plays any major roles in spore resistance, although it may prevent large molecules, such as antibodies, from accessing the more inner layers of the spores (56, 63, 64). In addition, the *C. difficile* exosporium has been shown to bind to host factors and help become internalized by epithelial cells (65).

## WHAT ARE SPORES RESISTANT TO AND HOW RESISTANT ARE THEY?

It is often said that spores are extremely resistant, and as noted above, the list of what they are resistant to is long (10), and includes (i) UV and visible light and ionizing radiation; (ii) wet heat, probably the most familiar of spores' resistance properties; (iii)

dry heat, desiccation, and high vacuum; (iv) a host of chemicals, including those that alkylate, oxidize, or hydrolyze DNA and proteins, including strong oxidizing agents, such as peroxides or hypochlorite, as well as aldehydes, acid, and bases; (v) enzymes that degrade cell wall PG (also known as "eat resistance"); (vi) high hydrostatic pressure (HHP), which is used by the food industry; and (vii) a number of less well studied agents, including high pressure or supercritical gasses, and 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 (66). 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 the effects of sporulation conditions on these spores' wet heat resistance (16, 67, 68). However, the effects of sporulation conditions on spores of many other species, including *B. subtilis*, have also been studied (14, 69–73).

Variables in sporulation conditions examined for their effects on spore resistance include (i) temperature, (ii) water activity, (iii) pH, (iv) divalent cation type and amount, (v) addition of agents that increase the reducing power of sporulation media, and 6) relative medium richness. Much of the effect of these variables on sporulation output are summarized in a relatively recent review on this topic (3). However, although 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 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 (73), which might be expected to alter spore wet heat resistance as well as resistance to predation and perhaps some chemicals, but this topic 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 these effects could certainly alter the divalent cations chelated with Pyridine-2,6-dicarboxylic acid (DPA), which is 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 medium is generally not followed up by quantitation of the levels of divalent cations in the spores produced, and whether they are in the spore core or spore outer layers, so this topic 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 topic 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 with 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.

### UV and Visible Light and Ionizing Radiation

Spores of *Firmicutes* are generally 10- to 30-fold more resistant to 254-nm UV radiation, which is the optimum UV wavelength for damaging DNA, than growing cells. The major cause of spore UV resistance is the saturation of spore DNA with the $\alpha/\beta$-type SASP mentioned above (12). All spore-forming *Firmicutes* genomes have 2 to 7 genes encoding these very homologous 60- to 80-residue proteins that in total make up $\sim$5% of total spore core protein, and these genes are considered members of the set

**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 | Data for: Growing cells (wild type)[a] | Dormant spores (wild type)[b] | Dormant spores (*spoVA*$^{2mob}$)[b] | Key resistance factors |
|---|---|---|---|---|
| Wet heat (93°C)[c] | <0.05 | 35 | >120 | Low core water, SASPs, transposon gene(s), coat |
| Dry heat (120°C)[c] | <5 | 170 | 200 | SASPs |
| UV at 254 nm[c] | <0.1 | 1.2 | 1.3 | SASPs, DNA repair, coat |
| NaOCl (2.5% available chlorine)[c] | <0.1 | 4.5 | 6.5 | Coat |
| $H_2O_2$ (11%)[c] | <0.5 | 205 | 280 | SASPs, coat, IM |
| HCHO (2.5%)[c] | <0.1 | 20 | >60 | SASPs, coat, IM |
| $HNO_2$ (200 mM)[c] | <0.1 | 15 | >40 | SASPs, IM |
| $C_{12}H_{27}N$ (1 mM)[c] | ND | 20 | >60 | IM |
| Desiccation[d,e] | <1 | >20 | ND[f] | SASPs |
| γ-Radiation (100 krads)[e] | 1 | 28 | ND[f] | SASPs, DNA repair |

[a]Data from reference 12 except where indicated.
[b]Data from reference 51 except where indicated.
[c]Data are time in minutes to kill 90% of the population.
[d]No. of freeze-thaw cycles.
[e]Data show % survivors.
[f]ND, not determined.

of genes essential in a spore former (5, 6). The $\alpha/\beta$-type SASPs are synthesized only in the developing spore core very late in sporulation and are stable in the dormant spore. However, these proteins are rapidly degraded when spore germination is complete, via a process initiated by a SASP-specific endoprotease (74, 75). The free amino acids generated in this degradation provide amino acids for the synthesis of most new proteins soon after germination is complete. The $\alpha/\beta$-type SASPs collectively saturate DNA in spores, and the core's low water content is essential for the saturation, as when spores lacking the SASP-specific protease complete germination and core water content rises to 80% of wet weight, much of the $\alpha/\beta$-type SASPs dissociate from the spore chromosome (76). Notably, the three regions of the $\alpha/\beta$-type SASPs that are the most highly conserved throughout evolution are two that directly bind DNA and the region with the SASP-specific protease recognition sequence (12).

Saturation of DNA with $\alpha/\beta$-type SASPs, either in spores or *in vitro* with purified proteins from a number of species, transitions DNA structure from a B to an A-like conformation (Fig. 2C) (77–79). This transition drastically alters the 254-nm UV photochemistry of the DNA from the generation of cyclobutane-type pyrimidine dimers (CPDs) and 6-4-bipyrimidine photoproducts (6-4PPs) in growing cells or DNA *in vitro* to a thyminyl-thymine adduct termed the spore photoproduct (SP) (Fig. 2B) in spores or DNA saturated *in vitro* with $\alpha/\beta$-type SASPs from either *Bacillus* or *Clostridium* species (80, 81). Notably, wild-type (wt) spores of several species are much more UV resistant than spores lacking ~85% of $\alpha/\beta$-type SASPs, termed $\alpha^-\beta^-$ spores, and UV irradiation of $\alpha^-\beta^-$ spores generates CPDs and most likely 6-4PPs. The large CaDPA depot in the spore core also is involved in spore UV resistance but sensitizes spore DNA to damage (82).

CPDs and 6-4PPs, as well as the 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 (Fig. 2D) as well as enzymes of nucleotide excision repair (NER) and base excision repair (BER) (83–85). 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 (85). Note that UV-treated spores, even if some are killed, can still germinate, which is 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 (86, 87). Since 254-nm UV forms SP and CPD with relatively similar efficiency, it is the error-free nature of SP repair compared with 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 wild-type spores, and the concomitant absence of other DNA repair proteins leads to even lower UV resistance (88). Thus, both $\alpha/\beta$-type SASPs and DNA repair are crucial for full spore resistance to UV at 254 nm. The need for both $\alpha/\beta$-type SASPS and DNA repair is also the case for resistance to UV at 222nm, 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 (89, 90).

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 (91, 92) and even more against 400 nm blue light (90). 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 to 1,100 nm (93, 94), and $\alpha/\beta$-type SASPs and DNA repair are again important in resistance to this treatment (95). The spore coat is also important in resistance to pulsed white light. Spores with severe coat mutations are killed more rapidly than wild-type spores, and this treatment also removes some coat proteins from spores (96). Intriguingly, spores exposed to pulsed near-infrared light (700 to 1,000 nm) showed only 2-fold enhanced resistance to its bactericidal effects compared with vegetative cells, which is a surprisingly small deviation in sensitivity and one that warrants further investigation (97).

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 (38, 98, 99), indicating that $\alpha/\beta$-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 (98), this topic needs a thorough investigation. As seen with UV radiation, ionizing radiation was found to generate DNA damage in spores, as evidenced by increased mutation levels in survivors (100). Consequently, it is not surprising that spore resistance to ionizing radiation resistance is increased by the presence of a number of DNA repair enzymes in spores. They act once germination is complete and include enzymes involved in recombination repair, BER repair, abasic site repair, and homologous and nonhomologous end joining (38, 101, 102). Notably, spore radiation resistance parallels the incorporation of cysteine into developing spores (103), whereas spores produced in cysteine-enriched media show increased resistance to nonionizing UV between 280 and 400 nm (70). However, the underlying reasons for apparent cysteine-associated resistance to ionizing and nonionizing radiations have not been established.

### 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 (e.g., 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, (ii) inactivation of one or more essential germination components, or (iii) breaking down the spores' IM permeability barrier so that CaDPA is released (16, 104–106); notably, CaDPA release from wet heat-treated spores of several species is well after spore death (104). Importantly, wet heat-treated spore populations, even if $\sim$95% is killed, can still exhibit percentages of germination of $>$50% (104, 106). However, only a few percentages of these germinated spores enter outgrowth and

almost all make minimal amounts of ATP or other high-energy intermediates (106). This work and other data (107, 108) have led to the suggestion that spore killing by wet heat is due to the inactivation of one or more crucial enzymes in the central metabolism in germinated spores—more on this later.

Pioneering work on spore wet heat resistance by Phil Gerhardt and Bob Marquis (109) established a number of additional features of spores that are important in their wet heat resistance, as follows: (i) the optimum growth temperature of the organism, with spores from organisms with higher optimum growth temperatures and having more intrinsically heat-resistant proteins, being more wet heat resistant (e.g., spores of the thermophile *Geobacillus stearothermophilus*); (ii) the degree of mineralization of the spore core and the specific cation(s) associated with DPA, with $Ca^{2+}$ being the cation giving most wet heat-resistant spores and with higher CaDPA levels in the core also generally giving more wet heat-resistant spores (note this effect can be difficult to separate from effects of elevated core CaDPA on core water content, as noted below); (iii) the sporulation temperature, with higher sporulation temperatures that still allow sporulation giving more wet heat-resistant spores; and most importantly, (iv) the water content in the spore core which ranges from 25% to 55% wet weight, in contrast to the usual 80% of wet weight in growing cells, with lower core water contents found in most wet heat resistant spores. Indeed, spores made at higher temperatures generally give spores with lower core water content.

One major question about spore core water is how the low spore water content is achieved late during sporulation. A partial answer to this question is that it is due to the developing forespores taking up CaDPA made in the mother cell very late in sporulation. Indeed, CaDPA uptake is paralleled by extrusion of some core water. In *B. subtilis* spores, this process reduces core water from 45% of wet weight to ~35% of wet weight (110). However, well prior to CaDPA uptake by the developing spore, (i) the volume of the spore core has already decreased significantly as has spore core water content (111) and (ii) soon thereafter, as much as 30% of the IM of developing forespores is extruded into the spore core and the spore core only fuses with the IM upon completion of spore germination (42). A major question then is how core volume is decreased in forespore 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 (52). In addition, it is now well appreciated that spores undergo what has been termed "maturation" after spores are released from the sporangium (112). This process can lead to large increases in resistance to wet heat and hypochlorite and is most likely due to increased cross-linking of coat proteins by enzymes in the coat (48, 49), 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 (110, 113), and many studies indicate that soluble core proteins are stable in wet spores to ~40°C higher temperatures than when heated in solution (107).

In addition to the mechanisms noted above, another very important mechanism protecting spores from wet heat is the saturation of spore DNA with the $\alpha/\beta$-type SASPs described in the section above. At elevated temperatures, both pyrimidine and purine bases in DNA can undergo cleavage of the glycosidic bond generating an abasic site *in vitro* or in growing cells (114), with depurination more rapid than depyrimidination, and both processes are faster at lower pH values (115). Notably, the repair of abasic sites is significantly error prone and generates mutations, as noted above (115, 116). Since spores of multiple species in water are quite resistant to temperatures of >75°C for significant amounts of time, 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 depurination is not observed, as there are minimal abasic sites in wild-type spores killed 99.9% by wet heat (114). In contrast, wet heat-treated $\alpha^-\beta^-$ spores killed to the same level as the wild-type spores had >20-fold higher levels of abasic sites, which also correlated with the frequencies of DNA strand breaks in these wet heat- $\alpha^-\beta^-$ treated spores (114). This prevention of depurination of DNA in wild-type spores is almost certainly due to the saturation of spore DNA by $\alpha/\beta$-type SASPs, as (i) these proteins block DNA depurination *in vitro* and (ii) an $\alpha/\beta$-type SASP variant that binds DNA poorly does not protect DNA from depurination *in vitro* and does not protect spores well against wet heat (117). DNA binding by $\alpha/\beta$-type SASPs also greatly inhibits deamination of cytosine residues in DNA (115), another potential mutagenic event. Indeed, the $\alpha/\beta$-type SASPs protect spore DNA from damage even at elevated temperatures, including at up to >100°C in a steam autoclave (16). Consequently, spore preparations killed by 90 to 95% by wet heat at various temperatures exhibit no increase in mutation frequency, in contrast to $\alpha^-\beta^-$ spores which (i) are significantly more sensitive to wet heat than wild-type spores (Table 1), (ii) accumulate high levels of mutants in spore populations killed by 90 to 95%, and (iii) become even less wet heat resistant if a crucial DNA repair protein such as RecA is also absent (85). In contrast, the loss of RecA has no effect on the resistance of wild-type spores to wet heat (85). Presumably, the protection of DNA by $\alpha/\beta$-type SASP binding is so great that spores die from other effects of wet heat well before there is any DNA damage. This other lethal wet heat damage is most likely to spore proteins, and a small amount of protein denaturation takes place during spore killing by wet heat, and prior to CaDPA release, which takes place in only dead, wet heat-treated spores. Following all CaDPA release, there is rapid denaturation of large amounts of spore protein, presumably because of the increased core water content once CaDPA has been released (104). As noted above, there is good evidence that the spore coat also is important in spore resistance, as spores with severely defective coats have significantly lower wet heat resistance. Why the coat is important in wet heat resistance is not completely clear, but it may be multifactorial, i.e., in addition to its rigidity influencing cortex-mediated core volume, it may be because it, along with CaDPA, is important for maintaining a relatively immobile IM environment, as is discussed below.

A more subtle effect on spore wet heat resistance is seen when wild-type spores are treated with wet heat applied either conductively or by ohmic (i.e., electrical resistance) heating (118). Notably, ohmic heating kills wild-type spores more rapidly than the same temperature applied to these spores by way of conductive heating. A possible clue to the mechanism of this effect came from demonstrating that $\alpha^-\beta^-$ spores were killed equally well by wet heat applied either conductively or ohmically. These data suggest that spores' high resistance to wet heat due to $\alpha/\beta$-type SASP-binding can be subverted by ohmic heat, as the ohmic treatment may weaken interactions between $\alpha/\beta$-type SASPs and DNA which are not extremely strong. Conceivably, dissociation of the proteins from DNA could lead to heat-induced damage on any naked DNA prior to $\alpha/\beta$-type SASP rebinding to DNA when spores are cooled. However, recent work has shown that ohmic heat killing of wt *B. subtilis* spores is not associated with increased mutagenesis (S. Singh, M. Ali, J. H. Mok, G. Korza, P. Setlow, and S. K. Sastry, unpublished results), so further work is needed to determine exactly how ohmic heat accelerates wild-type spore killing.

As a reminder that "nature always finds a way," a new mechanism of spore resistance to wet heat was identified a few years ago in spores of some extremely wet heat-resistant spores of *B. subtilis* and its close relatives that had survived in a plant processing foodstuffs at very high temperature (17, 18). The organisms giving these very heat-resistant spores had all acquired a transposon with an operon, termed *spoVA*$^{2mob}$, that was likely expressed only in the developing spore. Notably, the extreme resistance of these spores was largely due to a gene on this operon called *2duf*, denoting that it has two domains of unknown function, one being DUF421 (51). These heat-resistant spores had neither an elevated

CaDPA content nor a decreased core water content (19), indicating that something new was giving rise to the extremely high spore wet heat resistance, and this was likely 2Duf alone or together with some other gene product expressed on the *spoVA*$^{2mob}$ operon. From its primary structure, 2Duf seems most likely to be an IM protein. Indeed, while not found in a proteomic analysis of spores carrying *spoVA*$^{2mob}$ (119), a 2Duf orthologue was found in the *Bacillus cereus* spore IM proteome but not in growing cells (120, 121). Notably, spores with 2Duf are highly resistant not only to wet heat but also to several agents that either modify the IM or must cross it to damage molecules in the core, either proteins damaged by $H_2O_2$ or DNA damaged by nitrite or formaldehyde (51). It has been known for a number of years that the spore IM has a much lower permeability than the plasma membrane of a growing cell, even to molecules like methylamine or even water (41). This low spore IM permeability is due in part to the spore coat, the removal of which may release cortex pressure on the core such that the IM can expand very slightly, and to the high level of CaDPA of the spore core (51). Note that a lipid probe in the spore IM is largely immobile (41), and the lipid mobility and permeability of the IM are both increased significantly in spores that lack coats and CaDPA (122); perhaps free coordination sites of $Ca^{2+}$ in CaDPA bind to phospholipid headgroups on the inner IM leaflet and make the IM more rigid and less permeable. While the precise effect of 2Duf on IM structure is not well understood, recent results indicate that the presence of the *spoVA*$^{2mob}$ operon makes lipids in the IM more immobile and less permeable to agents that must enter the core to damage core components (51). In addition, the presence of 2Duf stabilizes IM germination proteins to wet heat (119) and makes IM germinant receptors more refractory to the heat activation that can potentiate spore germination (108).

The decreased IM permeability of spores containing 2Duf noted above seems likely to be the reason that spores with 2Duf have increased resistance to chemicals that must cross the IM to cause spore killing (51). 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 mode appears to be by the inactivation of one or more key enzymes needed for ATP generation in spore outgrowth (104–106, 123). Some of these proteins could be soluble enzymes for glycolysis, as spores have little if any of some enzymes of the tricarboxylic acid cycle (124, 125). 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 (125), and many of the enzymes for the latter are integral IM proteins (120). 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 idea is only a hypothesis, it is consistent with several other facts, including that (i) 2Duf does not provide spores with increased resistance to dry heat or UV radiation, of which both kill spores by DNA damage, and (ii) the viability of wet heat-treated spores of several species can be significantly increased by the 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, which is also the case for *B. subtilis* spores treated with hydrogen peroxide (105, 126). Clearly, further work is needed to fully understand in detail all the effects of 2Duf on the IM of spores 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 (121). Mutations in these two genes also reduced spore resistance to agents that must cross the IM to damage core components, including $H_2O_2$, nitrite, and formaldehyde. The loss of YetF or YdfS also had slightly decreased rates of L-valine germination and had stronger effects on AGFK germination. The effects of the *yetF* mutation were complemented by reintroduction of the *yetF* gene at the *amyE* locus, and introduction of *yetF* at *amyE* in a wild-type strain

increased spore wet heat resistance (121). Importantly, YetF and YdfS are predicted to be membrane proteins and have been found in the IM of spores, as was a YetF-GFP fusion (127, 128). While the CaDPA and core water content of *yetF B. subtilis* spores are identical to those of wild-type spores, the phospholipid composition of spores with and without *yetF* or *ydfS* was also examined, as work 13 years ago showed that changes in specific IM phospholipid levels could decrease *B. subtilis* spore resistance to wet heat and hydrogen peroxide (40). However, an analysis of IM phospholipid levels in spores with or without 2Duf, YetF, or YdfS found that there were no significant differences between the IM phospholipids in spores of these three strains (121, 123) While these latter results rule out a significant role for IM phospholipids in modulating effects of YetF and its homologs on spore resistance, the initial findings (40) of large effects of changes in IM phospholipid levels on spore wet heat and $H2O_2$ resistance also provide further support for the major role of the IM in determining rates of spore killing by these two agents.

The work on the effects of YetF and YdfS on spore resistance to date has focused on *B. subtilis* spores in which YetF is most likely present in higher levels in spores than that in vegetative cells, and it is perhaps higher than YdfS levels in spores. These two proteins are members of the DUF421 superfamily of proteins that are found in an enormous number of *Firmicutes* spore formers and which exhibit similar structures when AlphaFold models are compared (121). Even some non-spore-forming bacteria contain one or more members of this gene family, but to date, no 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 methods other than conductive means, with 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 (129), others have found no such additive effect (130–133), 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 treatment such is carrying out sonication of spores at an elevated temperature, a process termed thermosonication (TS) (134–138). TS kills spores of many species. With *B. subtilis* spores, the factors important in resistance to this treatment are most importantly the $\alpha/\beta$-type SASPs (137), although evidently TS treatment results in damage to the inner membrane and spore coat (135, 137, 138).

### 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 (139). As with wet heat, the $\alpha/\beta$-type SASPs 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 $\alpha/\beta$-type SASPs, and $\alpha^-\beta^-$ spores are more sensitive to dry heat that wild-type spores (116). However, unlike with wet heat, wild-type spore populations killed 90 to 95% by dry heat accumulate high levels of mutations (116, 140, 141). Consequently, DNA repair when spores begin outgrowth is also important because their dry heat resistance and mutations in major DNA repair proteins, such as RecA, greatly decrease spore dry heat resistance (85). The DNA damage generated by dry heat treatment of spores includes both transitions and transversions (142).

Wild-type spores are generally very resistant to desiccation alone and can survive multiple cycles of hydration and desiccation at moderate vacuum (~10 Pa) (143). The $\alpha/\beta$-type SASPs are a major factor in spore desiccation resistance at moderate vacuum, as $\alpha^-\beta^-$ spores are killed 30 to 75% by even one cycle of desiccation under moderate vacuum and then rehydration, with multiple cycles giving more and more killing; it is the processes of either desiccation or rehydration or both that give the killing of the $\alpha^-\beta^-$ spores, not the time in the desiccated state (143). Again, DNA damage seems to

be the mechanism of the killing of the $\alpha^-\beta^-$ spores by desiccation, as there is increased mutagenesis of the survivors and the presence of DNA repair enzymes in spores is important in desiccation resistance of $\alpha^-\beta^-$ spores. While wild-type spores survive desiccation at moderate vacuum, desiccation at high vacuum ($\leq$0.1 Pa) results in spore killing by DNA damage (27, 144). Again, the $\alpha/\beta$-type SASPs as well as DNA repair during spore outgrowth are most important in resisting spore killing by high vacuum (101, 142, 145, 146).

**Chemicals**

Spores can be killed by a wide range of chemicals, including a variety of DNA-damaging agents; some amines and alcohols; and oxidizing agents, aldehydes, acids, and bases (11, 147). For potential DNA-damaging chemicals that can alkylate, deaminate, or oxidize DNA, spore resistance to these agents is due to (i) the low IM permeability, as increases or decreases in IM permeability correlate with more rapid or slower spore killing, respectively (123, 126, 148); and (ii) DNA protection by $\alpha/\beta$-type SASPs, which is so effective that several peroxides, including hydrogen peroxide, do not kill wild-type spores by DNA damage but most likely kill them by oxidative damage to amino acid residues, in particular methionines, in one or more core enzymes (149–151). For example, even $\alpha/\beta$-type SASPs bound to DNA in spores can undergo oxidation of methionine residues by multiple peroxides, as can methionines in other core proteins, and these agents can also oxidize lysine $\gamma$-amino groups in core proteins to aldehydes (152). Presumably, it is by protein damage that wild-type spores are killed by these peroxides. However, the protein(s) damaged by oxidizing agents which leads to spore death is not known, although killed peroxide-treated spores can germinate, albeit slowly (153). Indeed, germinated hydrogen peroxide-killed spores do not accumulate ATP, as is the case with germinated wet heat-killed spores, and perhaps both agents target similar IM enzymes involved in oxidative phosphorylation (104, 123, 154). In contrast to the behavior of wild-type spores, at least hydrogen peroxide kills $\alpha^-\beta^-$ spores by DNA damage, and DNA repair in peroxide-treated outgrowing $\alpha^-\beta^-$spores is important in the resistance of these spores (85).

Larger, more hydrophilic potential DNA-damaging agents, especially if charged at neutral pH, do not kill spores by DNA damage. This group of chemicals includes strong oxidizing agents, such as hypochlorite, chlorine dioxide, or ozone, which do not kill spores by DNA damage as they presumably do not cross the IM, they do not kill $\alpha^-\beta^-$ spores more rapidly than wild-type spores, and the loss of DNA repair capacity has no effect on spore resistance to such agents (155, 156). In addition to the prevention of damage to spore core components by low IM permeability, major protection against these oxidizing agents as well as aldehydes (e.g., glutaraldehyde) is provided by the spore coat. Severe coat defects result in more rapid spore 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 (155–158). 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 during germination. However, the specific IM damage that causes this effect is not known but is not the oxidation of unsaturated fatty acids (126).

Another potential protective feature of the coat of spores of some species is that potential detoxification enzymes (e.g., catalase and superoxide dismutase) are present not only in the spore core but also in the coat (159, 160). The core enzymes play no role in spore resistance (161), even if their levels are greatly increased (162), 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 (163). 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 (164).

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 the loss of DNA repair, $\alpha/\beta$-type SASPs, or spore coats do not affect spore resistance to these pH extremes,

which is not really understood. However, findings with spore resistance to a strong alkali has exemplified some of the potential for misleading results that can arise in studies of spore killing by chemicals. 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 (165). Notably, given germinants, these alkali-treated spores rapidly release CaDPA in the initial step in spore germination, but they cannot degrade the cortex PG and complete the germination process (153). Presumably, the cortex-lytic enzymes are inactivated by strong alkali and lysozyme was a surrogate for these enzymes, as the treated spores were not truly dead. Another more insidious problem in assessing spore killing by chemical agents is that unless these are removed or fully inactivated, when spores are plated on nutrients to allow spore germination, toxic chemicals that have been carried over can rapidly kill the much more sensitive germinated spores. Sadly, this problem has led to more than one error in classifying a chemical agent as being able to kill dormant spores (166, 167).

### Eat Resistance

Sporulating cells spend a large amount of time and energy in making all the specific proteins that comprise the spore coat, for example, $\geq$80 proteins in *B. subtilis*, which together make up $>$50% of total spore protein. Given this large energy expenditure, one would presume that the coat provides some major benefit to spores, and indeed it does, as it deals with a major problem spores face when in soil or water which have many other types of organisms, including lower eukaryotes and predatory bacteria, for all of whom bacteria are their favorite "meal." The eukaryotic bacterivores ingest meals of soil or water, and enzymes in their digestive tract, most importantly PG hydrolases that lyse and kill any ingested bacteria, together with other hydrolases digest the meal to metabolites, allowing the predator to flourish. However, in this predator-rich environment, the spore can neither flee from its predators nor release some chemical defense but must stand its ground. The spore coat comes in here, as several studies have shown that intact spores of the model organism *B. subtilis*, a soil organism, are taken up readily either by the protozoan *Tetrahymena thermophilus* or the nematode *Caenorhabditis elegans*. However, the ingested spores are neither killed nor digested but rather are excreted no worse the wear from their brief sojourn in the digestive tract of these predators (53, 54). 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 (168, 169), 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, namely, *Myxococcus xanthus* and *Cupriavides necator*, that prey on other bacteria, including growing *B. subtilis* cells, but they cannot kill their spores (170). However, the mechanism of the resistance of *B. subtilis* spores to these predatory microbes is either not known (*M. xanthus*) or does not involve spore coats (*C. necator*) and may be due to dormancy and low IM permeability of the spores (170, 171). Note that spore dormancy and low IM permeability also preclude spore killing by antibiotics.

### 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 (172). However, HHP treatment can potentiate spore killing by triggering their germination at either moderate pressures (150 to 330 MPa) or much higher pressures (up to 900 MPa) (173, 174). While germinated spores can be killed by HHP, generally this method 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 than dormant spores (138, 175–178).

### Mechanical Disruption

Spores are much more resistant to mechanical disruption than growing cells, whether by sound waves (sonication) (134–138), by shaking with glass beads (179), or by piercing by silicon nanopillars (180). However, while studies of this type of spore killing have ruled out specific protective components, such as $\alpha/\beta$-type SASPs and most spore core proteins, the specific mechanisms involved in spore resistance to mechanical disruption have not been identified, and even the removal of much of the spore coat does not sensitize spores to mechanical disruption or piercing (179, 180).

### Miscellaneous

The past decade or so has seen the development and introduction of a fairly wide range of technologies aimed at inactivating spores in environments where traditional thermal and chemical approaches may not be appropriate (181). Various nonthermal gas plasma-based approaches have received considerable attention (182, 183), although knowledge of spore components damaged by nonthermal and low-pressure plasma samples and resistance counter measures is incomplete. However, the exposure of spores to cold atmospheric plasma was reported to damage the spore inner membrane and germination proteins, with the damage being caused by charged particles and reactive oxygen species as opposed to UV radiation (184). The configuration of plasma devices seems likely to modulate spore targets since a low-temperature nitrogen-oxygen plasma system was found to damage spore DNA via emission of UV-C and that SASPs and DNA repair enzymes conferred resistance in this regard (185). Another emerging approach concerns the application of supercritical gasses, usually $CO_2$ coupled with chemical modifiers for sterilizing heat-sensitive surfaces in the health care and food sectors (186, 187). Few studies have pursued the physiological basis for spore damage in this regard; however, spores exposed to supercritical $CO_2$ with peracetic acid were killed principally by damage to the inner membrane (188), with the coat and SASPs conferring various degrees of resistance to wet and dry spores, respectively. Finally, at the opposite end of the thermal spectrum, the exposure of spores to blast environment conditions, that is ~1,000°C for tens of milliseconds, resulted in efficient spore killing via damage to unknown spore proteins and perhaps the inner membrane (189). Evidently, there are limits to resistance, even for these most recalcitrant of microorganisms.

## CONCLUDING REMARKS

After more than 7 decades of intensive research driven in no small part by the quest for eradication, spores continue to confound and surprise. Just when progress in understanding the basis of resistance might have been in danger of stalling, new protein players in spore resistance (17–19, 121) are suddenly discovered, adding to the mosaic of features that collectively underpin spore recalcitrance. How were these players missed for so long in a busy field of research? If anything, recent events in the field demonstrate once again that spores do not easily give up their secrets. Indeed, an assessment of the literature, such as in this review, suggests that the current knowledge of spore resistance to a number of important stress factors frequently lacks granularity. Hence, as we move forward, the objective should be adding detail to "the damaged inner membrane," or the "undefined core protein," or more precisely defining what is meant by "coat disruption." Which proteins or lipids are involved? What is the effect at the nano and ultrastructural levels? What new and emerging techniques can we apply to probe the spore in such detail, and can we then use this knowledge to kill spores more efficiently? Clearly there is much left to be learned about spores, their resistance, and their ever-elusive Achilles' heels.

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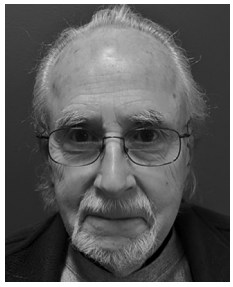

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