Spore Resistance Properties

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ABSTRACT Spores of various Bacillus and Clostridium species are among the most resistant life forms known. Since the spores of some species are causative agents of much food spoilage, food poisoning, and human disease, and the spores of Bacillus anthracis are a major bioweapon, there is much interest in the mechanisms of spore resistance and how these spores can be killed. This article will discuss the factors involved in spore resistance to agents such as wet and dry heat, desiccation, UV and γ-radiation, enzymes that hydrolyze bacterial cell walls, and a variety of toxic chemicals, including genotoxic agents, oxidizing agents, aldehydes, acid, and alkali. These resistance factors include the outer layers of the spore, such as the thick proteinaceous coat that detoxifies reactive chemicals; the relatively impermeable inner spore membrane that restricts access of toxic chemicals to the spore core containing the spore’s DNA and most enzymes; the low water content and high level of dipicolinic acid in the spore core that protects core macromolecules from the effects of heat and desiccation; the saturation of spore DNA with a novel group of proteins that protect the DNA against heat, genotoxic chemicals, and radiation; and the repair of radiation damage to DNA when spores germinate and return to life. Despite their extreme resistance, spores can be killed, including by damage to DNA, crucial spore proteins, the spore’s inner membrane, and one or more components of the spore germination apparatus.

The extreme resistance of spores of members of the Bacillales and Clostridiales orders is probably the property most closely associated with these spores. In the past, this extreme resistance contributed to claims for spontaneous generation and, in more recent years, has contributed to the applied importance of spores in a number of different areas including the following. (i) The food industry. Given that spores of a number of species are ubiquitous in the environment, they routinely contaminate foodstuffs. Since spores of many species are vectors for food spoilage and food-borne disease, the food industry commits significant resources to eliminating spores in order to make foods sterile, in particular to eliminate extremely dangerous spores such as those of Clostridium botulinum (1, 2). Indeed, many of the requirements for food sterilization regimens in the United States are designed to completely inactivate C. botulinum spores. (ii) The medical products industry. Just as in the food industry, spores present similar concerns in the medical products industry, including the manufacture of medical devices and parenteral drugs, again because of the involvement of spores in a number of human diseases. (iii) The health care industry. There is an increasing prevalence of disease due to Clostridium difficile in hospital and long-term nursing care facilities, largely because of the resistance of C. difficile spores and thus their persistence in patient care environments unless stringent environmental decontamination regimens are followed. (iv) Vaccine development. There is increasing interest in spores as carriers of proteins important as vaccines (3, 91), in large part because of spores’ extreme stability to normal and even extreme environmental conditions. This may allow the delivery of vaccines to areas where cold storage is difficult and is facilitated by utilizing the spore coat as a means to deliver immunogens. (v) Probiotics. Since spores are dormant, as such, they will not be probiotics. However, the administration of spores with their resistance to low pH conditions in

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the stomach is a route to effectively deliver potentially beneficial bacteria to the lower gastrointestinal tract (4, 91). Notably, it is the C. difficile spore’s resistance to stomach acidity that is the reason that the oral route is the major mechanism for C. difficile infection. (vi) Biological warfare. While the disease-causing potential of Bacillus anthracis is one reason that this organism has come to the fore as a biological weapon, in particular of bioterror (S. L. Welkos, unpublished data), the major reason for this organism’s visibility in this area is that B. anthracis spores are so resistant. This makes their dispersal either in water or as an aerosol relatively simple and ensures that these spores will persist in contaminated environments and will thus require stringent decontamination methods for their elimination.

Given the applied interest in spores, in large part because of their resistance properties, it is not surprising that there has been tremendous interest in the mechanisms of spore resistance. Most of this mechanistic work has utilized Bacillus subtilis spores because of the large number of B. subtilis strains with mutations in genes that may be involved in spore resistance. However, where it has been studied (5–7), work with spores of other Bacillus species has generally given results similar to those with B. subtilis. There has, however, generally been much less work done on mechanisms of resistance of spores of Clostridium species. In this report, the focus will be on work with B. subtilis spores, unless noted otherwise. However, relevant data from spores of other species or genera will be mentioned when available. References will largely be confined to the most recent work, while references to older work can be found cited in numerous past reviews on spore resistance properties (2, 8–15).

### OVERVIEW OF SPORE RESISTANCE

Spores exhibit greatly increased resistance to a large number of agents, including desiccation, freezing, thawing, elevated temperatures in either the wet or dry state, UV and γ-radiation, high pressures, and a huge number of toxic chemicals with a variety of nasty effects including oxidizing agents, alkylating agents, aldehydes, halogens, acids, and bases. Invariably, spores are much more resistant to these various agents than are growing cells of the same species (Table 1). However, spores of different strains, species, and genera can exhibit quite large differences in their resistance to various agents.

Spores are killed by damage to a number of different components, including DNA, the spore’s inner membrane (IM), proteins in the spore core, and likely other components as well (Table 2). Spores also utilize a variety of strategies to generate their extreme resistance (Table 3), including the maintenance of special outer layers to help protect sensitive spore components such as peptidoglycan (PG) from enzymatic attack and DNA from chemical attack. DNA in spores is also saturated with novel proteins that further protect the DNA against chemical attack and damage by wet heat. This novel DNA-protein complex in the spore is also important in protecting the DNA against UV and γ-radiation, as well as against dry heat and desiccation. Spores also contain a variety of novel enzymes that can rapidly repair DNA damage accumulated during spore dormancy, when spores return to life in the process of germination. Finally, the conditions inside the central spore core, where spore DNA and most spore enzymes are located, minimize damage due to agents such as wet heat and perhaps other treatments.

### Table 1: Resistance of spores and growing cells of B. subtilis to various agents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growing cells (wild type)</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>recA</td>
</tr>
<tr>
<td>None</td>
<td>82</td>
<td>35</td>
</tr>
<tr>
<td>Wet heat, 90°C (min)</td>
<td>&lt;0.05</td>
<td>18</td>
</tr>
<tr>
<td>Dry heat, 120°C (min)</td>
<td>35</td>
<td>18</td>
</tr>
<tr>
<td>Dry heat, 105°C (min)</td>
<td>95</td>
<td>–</td>
</tr>
<tr>
<td>Dry heat, 90°C (min)</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>H2O2 (15%) (min)</td>
<td>&lt;0.2</td>
<td>50</td>
</tr>
<tr>
<td>HCHO (25 g/liter) (min)</td>
<td>&lt;0.1</td>
<td>22</td>
</tr>
<tr>
<td>HNO2 (150 mM) (min)</td>
<td>&lt;0.2</td>
<td>100</td>
</tr>
<tr>
<td>NaOCl (50 mg/liter, pH 7)</td>
<td>&lt;0.1</td>
<td>55</td>
</tr>
<tr>
<td>UV-254 nm (kJ/m²)</td>
<td>36</td>
<td>330</td>
</tr>
<tr>
<td>Desiccation (#)</td>
<td>&lt;1</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

aData are at 23°C unless otherwise noted, and are from reference 13.

Core water content as % wet wt.

Time (min), radiation dose or the number of freeze-drying cycles to kill 90% of the population.

Vegetative cells were dried in sucrose and spores were dried from water.

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Setlow
As will be seen when spore resistance to specific agents is discussed below, often multiple mechanisms contribute to spore resistance to one agent (Table 3); for example, both DNA protection and DNA repair contribute to spore resistance to radiation. Similarly, specific individual resistance mechanisms often contribute to resistance to more than one type of agent (Table 3). For example, a spore’s outer layers contribute to resistance to both predation and many reactive chemicals. Together, all of these general mechanisms make spores one of the most resistant life forms known.

### ROLE OF SPORE STRUCTURE IN SPORE RESISTANCE

Spore structure plays a major role in spore resistance, as a number of the spore layers play specific roles in resistance. From the outside in, the various spore layers include the exosporium, coat, outer membrane (OM), cortex, germ cell wall, IM, and core (Fig. 1). The outermost exosporium is not present in spores of all species and is absent in *B. subtilis* spores. However, in spores of *B. anthracis* the exosporium may act as a permeability barrier restricting access of antibodies to antigens present in the spore coat (16). Other than this, there is no evidence that the exosporium plays any significant role in spore resistance. The spore coat contains a large fraction of total spore protein and acts as a permeability barrier restricting access of large molecules such as enzymes to potential sensitive targets in the spore’s more inner layers (A. Driks and P. Eichenberger, unpublished data). Consequently, the spore coat is responsible for protection against enzymes such as lysozyme that degrade peptidoglycan (PG), and thus for protection of spores against predation by bacteriovores (17, 18). The coats are also important in spore protection against a variety of biocidal chemicals, probably by reacting non-specifically with and detoxifying such chemicals before they reach more essential targets further within the spore (11, 14, 16, 19). This has been shown best by using *B. subtilis cotE* spores that lack the CotE protein essential for spore coat morphogenesis (16), as cotE spores have greatly decreased resistance to some chemical biocides including hypochlorite and nitrous acid (Table 1). The spore coats also contain some enzymes that can detoxify potential biocidal chemicals such as peroxides, and this can further increase spore resistance to such agents. In spores of some species, the coats may contain pigments that absorb strongly in the UV region, and there is suggestive evidence that such pigments can play a significant role in spore UV resistance (11, 20).

Underlying the coat is the OM, the role of which in spore resistance is not completely clear. The OM also can contain pigments, generally carotenoids that may play a role in spore UV resistance as noted above (11, 20). However, the possible role of the OM as a permeability barrier is not clear. In general, an intact OM is not seen in electron micrographs of spores, and the

### TABLE 2 Mechanisms of spore killing by various agents

<table>
<thead>
<tr>
<th>Mechanism of spore killing</th>
<th>Examples of agents that kill spores by this mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA damage</td>
<td>EtO, nitrite, formaldehyde, dry heat, UV and γ-radiation</td>
</tr>
<tr>
<td>Inner membrane damage</td>
<td>Hypochlorite, ClO₂, ozone, some peroxides</td>
</tr>
<tr>
<td>Inactivation of core enzymes</td>
<td>Wet heat, perhaps H₂O₂</td>
</tr>
<tr>
<td>Germination apparatus damage</td>
<td>Alkalies, dialdehydes</td>
</tr>
<tr>
<td>Unknown damage</td>
<td>High [HCl]</td>
</tr>
</tbody>
</table>

*Information is for spores of *B. subtilis* and is from references 2, 13, and 14.

1 Alkali treatment can often generate spores that appear to be dead, but that can be revived if artificially germinated with lysozyme. However, alkali can also kill spores completely, although the mechanism of this killing is not known.

2 High [HCl] causes spore rupture, but the primary reason for the rupture is not known.

### TABLE 3 Factors important in spore resistance to various agents

<table>
<thead>
<tr>
<th>Type of agent</th>
<th>Protective factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV radiation</td>
<td>DNA saturation by αβ-type SASP; DNA repair during spore outgrowth; low core water content; carotenoids in spore outer layers</td>
</tr>
<tr>
<td>γ-Radiation</td>
<td>DNA saturation by αβ-type SASP; DNA repair during spore outgrowth</td>
</tr>
<tr>
<td>Desiccation</td>
<td>DNA saturation by αβ-type SASP; DPA</td>
</tr>
<tr>
<td>Dry heat</td>
<td>DNA saturation by αβ-type SASP; DNA repair during spore outgrowth; DPA; perhaps divalent metal ion content</td>
</tr>
<tr>
<td>Wet heat</td>
<td>DNA saturation by αβ-type SASP; DPA level; low core water content; sporulation conditions including temperature; divalent metal ion content; sporulation temperature optimum</td>
</tr>
<tr>
<td>Genotoxic chemicals</td>
<td>DNA saturation by αβ-type SASP; DNA repair during spore outgrowth; low core water content</td>
</tr>
<tr>
<td>Oxidizing agents</td>
<td>Spore coat protein; low permeability of spores’ inner membrane; DNA saturation by αβ-type SASP; detoxifying enzymes in spore’s outer layers</td>
</tr>
<tr>
<td>Dialdehydes</td>
<td>Spore coats</td>
</tr>
<tr>
<td>Disinfectants</td>
<td>Spore coats, perhaps cortex and inner membrane structure</td>
</tr>
<tr>
<td>Acids and alkali</td>
<td>Not understood</td>
</tr>
<tr>
<td>Plasma</td>
<td>Spore coat?; DNA saturation by αβ-type SASP; not yet thoroughly studied</td>
</tr>
<tr>
<td>Bacteriovores</td>
<td>Spore coat</td>
</tr>
</tbody>
</table>

*Information is for spores of *B. subtilis*, and is from references 2, 5, 6, 11, 17, 18, and 50.

*Not all protective factors are important in protecting against all chemicals of any particular type.*
disruption of the spore coat layer by mutations in key coat protein genes is sufficient to allow lysozyme to attack spore PG layers below the OM. However, the specific role of the OM in dormant spore resistance remains an open question, although this membrane does play an essential role in spore formation. Underlying the OM are two PG layers, first the spore cortex and then the thinner germ cell wall, each with PG of slightly different structures (D. L. Popham and C. B. Bernhards, unpublished data). While both of these layers are essential for spore viability, and the cortex undoubtedly is essential for some of the novel properties of the core (see below), these two layers are not known to play any active role in spore resistance.

Under the germ cell wall is the spore’s IM. While the lipid composition of the IM is not particularly unusual (21), the IM itself has some very novel properties (11). In particular, (i) lipids in the IM are largely immobile; (ii) the IM has a much higher viscosity than the germinal spore’s plasma membrane; and (iii) the IM’s passive permeability to small molecules is extremely low, even for molecules such as methylene and water (11, 22–24). While the reason(s) for these novel properties of the IM is not known, these properties, especially the IM’s relative impermeability, seem likely to be important in spore resistance to some biocidal chemicals by restricting these chemicals’ access to targets in the spore’s central core. Indeed, damage to the IM appears to be the mechanism by which a number of oxidizing agents kill spores, although the nature of this damage is unknown (11, 14). The novel properties of the spore’s IM are lost when spores complete germination.

The final spore layer is the central core, which has a number of novel features that appear to play many roles in spore resistance (11, 12, 14). These include (i) the core’s low water content (25 to 55% of wet weight), important in spore wet heat resistance; (ii) the high level of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (Fig. 2) in a 1:1 complex with various divalent cations, generally mostly Ca$^{2+}$, and important in spore resistance to some DNA-damaging agents and in maintaining spore dormancy; and (iii) high levels of a group of novel proteins, the α/β-type small, acid-soluble spore proteins (SASPs) that saturate spore DNA and protect it from damage due to many genotoxic chemicals, desiccation, dry and wet heat, and UV and γ-radiation. The α/β-type SASPs are synthesized only late in sporulation within the developing forespore, and the genes for these 60 to 75-amino-acid (aa) proteins are transcribed by RNA polymerase with a forespore-specific sigma factor, σ$^{C}$; Spores of all Clostridiales and Bacillales species contain α/β-type SASPs, with these proteins encoded by multiple monocistronic genes. The amino acid sequence of α/β-type SASPs has been tremendously conserved throughout evolution, most likely because of the importance of these proteins’ structure when bound to DNA that dramatically affects the structure of the protein-bound DNA (25). The core’s low water content also ensures that α/β-type SASPs remain bound to DNA in the dormant spore even though the affinity of these proteins for DNA is not extremely high. When spores germinate and then begin to outgrow, there is significant dissociation of α/β-type SASPs from DNA and the free protein is rapidly degraded, with the degradation initiated by a SASP-specific endoprotease. Interestingly, if the affinity of α/β-type SASPs for DNA is too high, the degradation of the α/β-type SASPs during spore outgrowth is not efficient, as some of these proteins remain bound to DNA. This generally results in the death of the

![Figure 1](https://doi.org/10.1128/microbiolspec.TBS-0003-2012.f1)

**FIGURE 1** Schematic structure of a *Bacillus* spore. Sizes of various layers are not drawn to scale; in many species, several different coat layers can be seen; spores of some species do not have an exosporium. doi:10.1128/microbiolspec.TBS-0003-2012.f1

![Figure 2](https://doi.org/10.1128/microbiolspec.TBS-0003-2012.f2)

**FIGURE 2** Structure of dipicolinic acid (DPA). Note that, at physiological pH, the two carboxyl groups will be ionized and the resultant carboxylate groups can chelate divalent cations. doi:10.1128/microbiolspec.TBS-0003-2012.f2
germinated spore. As is probably not surprising, expression of αβ-type SASPs in growing bacteria causes rapid cessation of cell growth as well as a loss in cell viability, and this effect is being investigated as a novel antibacterial therapy (26).

**SPECIFIC SPORE RESISTANCE PROPERTIES**

**Radiation Resistance**

**UV radiation**

Spores are 20- to 50-fold more resistant to UV radiation than growing cells (11) (Table 1). The magnitude of this difference depends on the species and on the UV wavelength. Not surprisingly, since DNA is the target of UV damage, 254-nm radiation is most effective in killing spores; longer and shorter wavelengths are also effective but require higher fluences than at 254 nm. There are also some differences in DNA UV photochemistry at different wavelengths (see below). Two major factors are responsible for spore UV resistance (Table 3): (i) a change in DNA’s UV photochemistry due to the binding of αβ-type SASPs; and (ii) DNA repair during spore outgrowth, with this repair catalyzed in part by spore enzymes (11, 27). In addition to these major resistance factors, pigments in the outer layers of some spores may be important in shielding spores from UV damage, and spores’ huge DPA depot also influences their UV resistance, as can be seen with spores that lack DPA (Table 1). Spores of several species can also be killed by high-intensity 405-nm visible light, although they are significantly more resistant than the corresponding vegetative cells (28, 29). However, nothing is yet known about factors important in spore resistance to 405-nm radiation.

UV irradiation of DNA at 254 nm in vitro or in growing bacteria generates a variety of photoproducts, including cyclobutane dimers (CPDs) (Fig. 1) between adjacent pyrimidines in the same DNA strand, as well as 6-4 adducts (64PP), again between adjacent pyrimidines (11). Both types of photoproducts are potentially lethal but can be repaired by a variety of mechanisms, many of which are significantly error prone. In contrast, UV irradiation of spores generates little if any CPD or 64PP, but rather a thyminyl-thymine adduct termed the spore photoproduct (SP) (Fig. 3). The SP is formed in spore DNA with a quantum efficiency not particularly different from that for CPD and 64PP formation in growing cells. However, SP formation in spores is a much less lethal photoproduct than CPD or 64PP because of its relatively error-free repair (see below). Interestingly, the photochemistry of DNA in spores changes somewhat at longer UV wavelengths, as some CPDs are formed at wavelengths >280 nm, although photoproduct formation at longer wavelengths requires much higher fluences than at 254 nm. There have also been studies using intense white light (200 to 1,100 nm) for spore inactivation (30), but studies on the mechanisms of spore resistance to and killing by such a treatment have not yet been done.

The major reason for the formation in spores of SP rather than CPDs and 64PPs upon UV irradiation is the saturation of spore DNA by the αβ-type SASPs that changes DNA from a B-conformation to a structure between that of A and B-DNA, resulting in a change in the DNA’s photochemistry (11, 25). Spores lacking the majority of their αβ-type SASPs (termed αβ− spores) no longer have sufficient amounts of these proteins to saturate their DNA and are much less resistant than wild-type spores to 254-nm UV radiation (Table 1) as well as to other UV wavelengths including solar UV (11, 31). Irradiation of αβ− spores by 254-nm radiation also produces large amounts of CPD and 64PP and much lower levels of SP. The spore’s low water content and high level of DPA both contribute to spores’ novel DNA photochemistry, and this has been duplicated in vitro with purified components (11). Although slight changes in core water content do not affect spore resistance to 254-nm UV, spores with elevated core water content are more sensitive to environmental UV radiation of >280 nm (31). The structure of the αβ− type SASP-DNA complex has been determined at high resolution, and analysis of this structure has indicated why UV irradiation of spores generates SP and not CPD or 64PP (25). The precise nature of the sporulation medium can also alter

**FIGURE 3** Structures of major photoproduct formed in growing cells (CPDs) and dormant spores (SP). The structures shown are as if these were formed between adjacent bases, with the nitrogen normally linked to the sugar in nucleosides shown with a hydrogen atom attached. The CPD shown is the major one, formed between two adjacent thymidine residues on the same DNA strand, although CPDs can also form between two adjacent cytidine residues and between adjacent cytidine and thymidine residues. SP is formed only between adjacent thymidine residues. doi:10.1128/microbiolspec.TBS-0003-2012.f3
spores’ resistance to UV radiation between 280 and 400 nm somewhat; in particular, the presence of potential radioprotective agents in the medium such as cysteine can result in spores with elevated resistance to UV as well as to γ-radiation, although these effects are eliminated if spores are first decoated [32]. There is also one report that a spore’s Mn level is important in its UV resistance [33], but the mechanism of this effect has not been studied in detail.

The altered UV photochemistry of spore DNA alone is just one part of spores’ elevated UV resistance, as SP is potentially a lethal photoprotein. The second major factor in spore UV resistance is the repair of UV damage to DNA during spore outgrowth. This repair is catalyzed by multiple independent enzymes/ enzyme systems including recombination repair, nucleotide excision repair, repair of abasic sites, and SP-specific repair [11, 12, 34, 35]. The first two repair systems use enzymes that are also involved in the repair of CPDs and 64PPs in growing cell DNA. However, repair of abasic sites and SP in spore DNA can use spore-specific enzymes, with SP repaired by spore photoproduct lyase (Spl), an S-adenosylmethionine (SAM)-dependent enzyme that monomerizes SP back to two thymine residues by using a radical-SAM mechanism [11, 12, 36, 37]. The spl gene is expressed only in the developing spore under control of the same RNA polymerase sigma factor, σC, that directs transcription of genes that encode α/β-type SASPs.

While most studies of the effects of α/β-type SASPs on spore UV resistance have been performed with spores of B. subtilis, the α/β-type SASPs are also a major factor in the UV resistance of Clostridium perfringens spores [38, 39]. Indeed, a C. perfringens α/β-type SASP can largely restore the UV resistance of α/β- B. subtilis spores, as well as their resistance to at least one genotoxic chemical [40]. Clotroclial α/β-type SASPs also have the same effects on DNA photochemistry in vitro as do the homologous B. subtilis proteins [11].

γ-Radiation
Spores are also more resistant to γ-radiation than are growing cells. Again the α/β-type SASPs contribute to spore γ-radiation resistance, although precisely how is not known. Repair of γ-radiation damage during spore outgrowth is also an important factor in spore γ-radiation resistance [41–43]. As with UV damage in spores, multiple enzymes are involved in the repair of γ-radiation damage to spore DNA, and at least some of these enzymes are spore-specific, including enzymes involved in repair of double-strand breaks in DNA. One area that has not been well studied in spore γ-radiation resistance is the precise spectrum of DNA damage generated by γ-radiation in spores, in comparison with γ-radiation damage generated in growing cells and in spores lacking α/β-type SASPs.

Chemical Resistance
Factors involved in spore resistance to chemicals
Spores are much more resistant than growing cells to a host of toxic chemicals, including aldehydes, oxidizing agents, alkylating agents, acids, and bases (Table 1) [2, 1, 12]. A number of factors are important in spore resistance to chemicals (Table 3), including (i) detoxifying enzymes in spore coats and/or exosporia; (ii) nonspecific detoxification by spore coat components; (iii) low permeation rates of toxic chemicals up to and through the spore’s IM; (iv) protection of DNA against chemical attack by α/β-type SASP binding; and (v) repair of chemically induced DNA damage during spore outgrowth.

Detoxifying enzymes in spore outer layers
Spores of several species have enzymes in their outer layers that can potentially detoxify toxic chemicals, including catalase to destroy hydrogen peroxide and superoxide dismutase (SOD) to eliminate superoxide [16, 44]. There is some evidence that at least SODs in spore outer layers are important in B. anthracis spore pathogenicity [45], perhaps by minimizing spore killing by superoxide generated inside host cell compartments. Enzymes such as catalase and SOD and others are also important in the resistance of growing cells to oxidative stress. However, while these enzymes are present in the spore core, these core enzymes play no role in dormant spore resistance, presumably because core enzymes are generally inactive due to the low core water content and protein immobility in the spore core [11, 14, 46].

Nonspecific detoxification of chemicals by spore outer layers
The presence of an intact spore coat is a major factor in spore resistance to many toxic chemicals including halogens, larger oxidizing agents, and aldehydes, and cotE spores are often more sensitive to such chemical biocides (Table 1). The reasons for this are not clear, but this is either because the rate of permeation of many of these molecules through the coats to more sensitive layers further within the spore is slow, or the huge amount of coat protein reacts with and detoxifies reactive chemicals before they reach the inner membrane [11, 14, 16]. At present, it is not completely possible to
decide between these possibilities—indeed, both of these explanations may be correct. Spores of at least one species also contain significant levels of a polycyclic terpenoid in outer spore layers that may serve to detoxify oxidizing agents (19).

Low permeability of the spore’s IM

Much work has shown that the spore’s IM has extremely low permeability to small molecules, even including water (11, 24). Thus, the IM’s permeability to potential DNA-damaging chemicals would be expected to be low. Indeed, there is significant evidence that changes in the permeability of the spore IM by sporulation at different temperatures result in spores with changes in sensitivity to DNA-damaging agents that parallel changes in IM permeability (11, 14, 22). For at least some chemicals, in particular hydrogen peroxide and low-molecular-weight DNA alkylating agents, the coats even play a minimal role in protection against such agents (11, 22).

DNA protection by α/β-type SASP binding

In addition to protecting against UV and γ-radiation, the saturation of DNA by α/β-type SASPs also protects spore DNA against a number of genotoxic chemicals, including hydrogen peroxide, nitrous acid, and formaldehyde (Table 1) (2, 11, 14). Invariably, αβ– spores are more sensitive to these agents than the latter chemical agents (Table 1). Protection against genotoxic chemicals is also given by αβ– type SASPs in C. perfringens spores (2, 11, 14, 39). As a consequence of the protection of DNA by αβ– type SASPs against genotoxic oxidizing agents such as hydrogen peroxide (H2O2), such agents do not kill spores by DNA damage, and loss of much DNA repair capacity by a recA mutation does not decrease otherwise wild-type spores’ resistance to H2O2 (Table 1). However, H2O2 does kill αβ– spores via DNA damage. Presumably, the protection of DNA against H2O2 in wild-type spores by αβ– type SASP binding is so strong that damage to some other spore component kills spores. Indeed, proteins important in repair of oxidative damage to DNA in growing cells, such as MutT and RecA, have no protective effects against H2O2 in spores (Table 1) (47). Formaldehyde and nitrous acid do kill wild-type spores by DNA damage, but αβ– spores are much more sensitive to these agents (Table 1).

It was initially surprising that αβ– type SASP binding does not protect spores against alkylating agents such as ethylene oxide (EtO) gas or ethyl methanesulfonate. However, αβ– type SASPs also do not prevent DNA alkylation in vitro. Ultimately, the determination of the αβ– type SASP-DNA structure at high resolution showed that DNA groups sensitive to alkylation are not at all shielded in the αβ– type SASP-DNA complex, as these sensitive groups are in the DNA’s major groove and αβ– type SASPs bind in DNA’s minor groove (23).

Repair of DNA damage

While in most cases chemical biocides do not kill spores by DNA damage, this is the case for some chemicals, including formaldehyde and nitrous acid as noted above. For these agents, DNA repair in spore outgrowth is also an important component of spore resistance to such agents, as shown by recA spores’ increased sensitivity to these two chemicals (Table 1). Enzymes present in dormant spores are also important in repairing damage caused by oxidizing agents that can be generated during spore germination and outgrowth (11, 14).

Mechanisms whereby chemical biocides kill spores

In addition to multiple mechanisms used by spores to resist chemical biocides, there are also multiple ways in which chemical biocides kill spores (Table 2), including (i) DNA damage; (ii) damage to the spore’s IM; (iii) damage to one or more key spore core enzymes; (iv) damage to the spore germination apparatus; (v) breaching all spore permeability barriers; and (vi) unknown mechanisms.

DNA damage

As noted above, some chemical biocides kill spores by DNA damage, including formaldehyde and nitrous acid, as well as alkylating agents such as EtO. However, for at least some potentially genotoxic chemicals, spore DNA is so well protected that damage to other spore components is how spores are killed.

IM damage

A large number of oxidizing agents kill spores by causing some kind of damage to the spore’s IM, such that this membrane readily ruptures when spores germinate, leading to rapid spore death (2, 11–14). However, the precise nature of this damage is not known, although it is not oxidation of unsaturated fatty acids. This likely IM damage can also sensitize spores to killing by other treatments such as wet or dry heat or desiccation (11, 14, 48). Indeed, mild pretreatment of spores with oxidizing agents potentiates DPA release from these spores when they are given a normally sublethal heat treatment (11–14).
Damage to key spore enzyme(s)
Several small oxidizing agents, \( \text{H}_2\text{O}_2 \) being the best studied, do not kill spores by DNA damage, yet they readily penetrate the spore core (11, 14). These agents cause significant inactivation of sensitive enzymes in the core, and in a few cases this enzyme inactivation precedes spore killing. While it is thus tempting to speculate that inactivation of one or more key spore core enzymes is the mechanism by which \( \text{H}_2\text{O}_2 \) and a few other peroxides kill spores, this has not been proven, and a key spore enzyme whose inactivation by \( \text{H}_2\text{O}_2 \) might lead to spore death has not been identified.

Damage to the spore germination apparatus
While there is no question that some chemical agents can inactivate one or more essential germination components, it is important to note that, in many cases, this type of defect can often be bypassed. In other words, spores killed by such a mechanism may be only conditionally dead and can be revived given proper treatment. The best-studied example of this phenomenon is spores treated with \( \text{NaOH} \) that appear dead, since the \( \text{NaOH} \) has inactivated the lytic enzymes needed to complete spore germination (11, 14). However, when plated with small amounts of lysozyme, these \( \text{NaOH} \)-treated spores exhibit normal viability. Other chemicals that also have effects on spore germination include some peroxides and dialdehydes. However, the specific targets of these agents have not been identified.

Breaching all spore permeability barriers
Strong acids have a remarkable sporicidal effect. When spores of many species are rapidly mixed with high concentrations of mineral acids, the spores undergo what is called “acid-popping,” in which the spores appear to rupture violently, releasing all spore contents (2, 11, 14). The cause of this dramatic effect is not well understood, but may well tell us something about the forces that maintain the intact spore structure. Interestingly, the silicon layer present in the outer layers of spores of some \textit{Bacillus} species, but not \textit{B. subtilis}, is important in protecting spores against killing by acid (49).

Unknown factors
A huge number of chemicals have been tested and found to have at least some sporicidal efficacy (11, 14). Not surprisingly, there is minimal if any knowledge of the mechanism of spore killing by many of these chemicals.

Heat Resistance
Spores are much more resistant to high temperatures than growing cells, both in the dry and wet states (11) (Table 1). In water, spores are resistant to 40°C higher temperatures than are growing cells of the same species, and to \( \sim 30 \)°C higher temperatures when dry. While some of the factors in spore resistance to wet and dry heat are identical (Table 3), the mechanisms of spore killing by wet and dry heat are different (Table 2), with protein damage likely killing spores exposed to wet heat, while DNA damage is a major mechanism whereby spores are killed by dry heat (5, 6, 11, 50–52).

Dry heat
The major known mechanism that protects spores from dry heat is the saturation of spore DNA with \( \alpha\beta \)-type SASPs, and, as expected, \( \alpha\beta^- \) spores have greatly decreased dry heat resistance. This is actually similar to the dry heat resistance of growing cells (Table 1) (11). However, even \( \alpha\beta^- \) SASP saturation of spore DNA is not sufficient to prevent dry heat from killing spores by DNA damage, with at least some of this damage being depurination. Consequently, DNA repair during spore outgrowth, at least some of which is RecA dependent, is also an important factor in spore dry heat resistance (Table 1), and a number of proteins important in this repair have been identified (11, 12, 53). Spores’ large DPA depot is also important in protecting spores against dry heat (54) (Table 1); however, the mechanism of this effect is not clear. The specific divalent ions chelated to DPA may also be important in spore resistance to dry heat, with Mn\(^{2+} \) being a cation whose level may be particularly important, at least with spores of some species (11, 32, 55). However, Mn\(^{2+} \) levels appear to play no role in spores’ \( \gamma \)-radiation resistance. Spores can also be sensitized to dry heat treatment by pretreatment with oxidizing agents (48).

Wet heat
Resistance to wet heat is probably the property most associated with spores, as spores of some species are resistant to \( \geq 100 \)°C for extended periods of time (2, 11, 12). A large number of factors are involved in spore resistance to wet heat (Table 3), including (i) DNA saturation with \( \alpha\beta^- \)-type SASPs; (ii) DPA; (iii) divalent metal ion content and identity; (iv) growth temperature optimum of the strain; (v) sporulation temperature; (vi) solid versus liquid sporulation medium; (vii) core water content; and (viii) additives present in the solution, in particular the pH, during wet heat treatment. There is also
significant evidence that wet heat resistance varies considerably between individuals in spore populations, although the reason for this heterogeneity is not known (see below).

**α/β-type SASP**
Wet heat does not kill spores by DNA damage (Table 1), which is somewhat surprising since the temperatures to which spores are resistant would be expected to lead to significant DNA damage, specifically depurination. This indicates that there must be significant protection of DNA against wet heat damage in spores, and this is due to the saturation of spore DNA with α/β-type SASPs in spores of both Bacillus and Clostridium species (Table 1) (2, 11, 39, 40). Presumably, the protection of spore DNA due to α/β-type SASP binding is so great that damage to some other spore component is what kills spores. Indeed, spores lacking α/β-type SASPs are killed at lower temperatures than wild-type spores and are killed by DNA damage (Table 1) (11). Much of the wet heat damage in αβ- spores is base loss likely via depurination, and α/β-type SASP binding has been shown to block depurination of DNA in vitro (11).

**DPA**
DPA may play no direct role in spore resistance to wet heat, but it certainly plays an indirect role, as when DPA is not accumulated in the spore core, the core’s water content does not decrease as much as in DPA-replete spores (11, 54) (Table 1). This is also the case in DPA-less C. perfringens spores (56). Since core water content is a major factor in spore wet heat resistance (see below), anything that results in increased spore core water content will decrease spore wet heat resistance. Consequently, DPA-less spores of both Bacillus and Clostridium species have higher core water content and significantly reduced wet heat resistance (Table 1) (11, 56).

**Divalent metal ions**
The spore core’s DPA depot is present primarily as a 1:1 chelate with divalent ions. The most common ion chelated with DPA in spores is Ca²⁺, although other divalent cations can be substituted, and even monovalent cations can replace the divalent cations. The precise type of cation associated with DPA has a significant effect on spore wet heat resistance, with Ca²⁺ generally giving the highest resistance and monovalent cations the lowest (4, 11, 57). There are also reports that altering the levels of some DPA-associated divalent cations, in particular Mn²⁺, can influence the wet heat resistance of spores in some but not all species, although the reason for this effect is not clear (2, 32, 55, 57).

**Growth temperature optimum of the strain**
It has long been known that spores of thermophiles have higher wet heat resistance than spores of mesophiles, with the latter generally being more wet heat resistant than the spores of psychrophiles (2, 11). The major reason for this effect is most likely that proteins in organisms that grow optimally at high temperatures are generally more thermostable than proteins in organisms that grow optimally at lower temperatures. Given that it appears most likely that it is damage to one or more key proteins that results in spore killing by wet heat (see below), it will thus generally require higher temperatures to inactivate a thermophile’s protein in spores. It is also notable that enzymes in spores exhibit resistance to ~40°C higher temperatures than do the same enzymes in vitro.

**Sporulation temperature**
Within a temperature range at which sporulation remains reasonably efficient, sporulation of a variety of different species and strains at higher temperatures results in spores with higher wet heat resistance than that of spores produced at lower temperatures (2, 11, 40, 58–63). The major factor responsible for this effect appears to be spore core water content, which is lower in spores produced at higher temperatures, and there is a reasonably coherent inverse relationship between core water content and spore wet heat resistance (see below). While other spore properties may also change depending on the sporulation temperature, no changes other than to core water content have been directly correlated with changes in levels of spore wet heat resistance.

**Solid versus liquid sporulation**
Sporulation at the same temperature on solid or liquid media also results in spores with slightly different wet heat resistance, and spores’ wet heat resistance is also altered by specific characteristics of solid sporulation media (64, 65). However, the reasons for these effects are not known.

**Core water content**
The water content in the spore core is probably the major factor in spore wet heat resistance, certainly for values between ~30 and 50% of core wet weight as water (11). The presumption is that a low core water content results in reduced molecular mobility of core
proteins and thus elevated protein resistance to thermal inactivation. Indeed, protein mobility in the dormant spore core is extremely low, as a normally soluble core protein, green fluorescent protein, is immobile in dormant spores, while its diffusion coefficient increases >4 orders of magnitude when spores complete their germination (46). There are, however, a number of unknowns about spore core water content, including (i) how core water content is lowered during spore formation—the spore cortex structure and the SpmA/B and DacB proteins play roles in determining spore core water content (11, 66–68), but how is not known—and (ii) how much spore core water is free water and how much is bound water; available evidence suggests that there is normally very little free water in the spore core, but this is based on only a very few measurements.

**Additives present during wet heat treatment**

There is an extensive older literature on the effects of additives on spore killing by wet heat, with pH being one variable that has been extremely well studied (2, 69). Thus pH values above 8 and below 6 during wet heat treatment decrease spores’ wet heat resistance, with larger effects as the pH diverges further from these values. There is also a recent report that several antimicrobial peptides can decrease spores’ wet heat resistance, although this effect may be species specific (70).

**Mechanism of wet heat killing of spores**

While dry heat kills spores by DNA damage, this is not how wet heat kills spores (Tables 1 and 2). Indeed, wet heat-treated spore populations accumulate no mutations and no DNA damage, and DNA repair defects, including a recA mutation, do not sensitize spores to wet heat (Table 1) (2, 11). Analysis of the kinetics of spore killing by wet heat indicates that release of the spore’s DPA takes place after spore killing (5, 6, 50). However, spore killing is paralleled and even preceded by damage to spore core proteins, including denaturation. These results suggest that wet heat kills spores by damage to one or more key spore proteins. However, the identity of these key proteins has not been established. Spores given a sublethal wet heat treatment also commonly exhibit slow germination, most likely because of inactivation of cortex-lytic enzymes essential for completion of spore germination (71). However, inactivation of cortex-lytic enzymes alone is not how wet heat treatment kills spores, since there is minimal if any recovery of wet heat-treated spores by subsequent lysozyme treatment.

**Heterogeneity in spore wet heat resistance**

As is probably not surprising, there is significant evidence that the wet heat resistance of individual spores in a population is quite heterogeneous (14, 49, 51, 52, 72–76). The most striking evidence for this is the analysis of the behavior of individual spores in water incubated at elevated temperatures when the level of spore DPA and the state of spore proteins is monitored throughout the incubation. Strikingly, DPA release from an individual spore incubated at 80 to 90°C takes only a few minutes, but begins only after an extremely variable lag period of minutes to hours in individual spores. Unfortunately, the factors that determine the length of this lag period prior to DPA release at elevated temperatures are not known, although DPA release is preceded by changes in spores’ protein spectrum that suggest there is some protein denaturation just prior to fast DPA release (5, 51, 52). Presumably, the spores with the longest lag periods prior to initiation of DPA release at elevated temperatures are the most wet-heat-resistant spores in populations. That spore populations do indeed have some spores with much higher levels of wet heat resistance has also been shown directly by isolation of superdormant spores as 1 to 2% of spore populations and demonstration that these spores have higher wet heat resistance than the general spore population (73). These latter spores also had a lower core water content than the spore population as a whole.

**Miscellaneous Spore Resistance Properties**

**High pressure**

Spores are much more resistant than growing cells to extremely high pressures (HPs), and they are also germinated by HP (77). The germination of spores, in particular, the release of spores’ DPA, is an essential step leading to spore inactivation by HP, which is most often by an elevated temperature (77–80). The reason for spores’ high resistance to HP is not known, but this does not require either α/β-type SASPs or an intact spore coat. The mechanism by which HP alone kills spores is also not known; as noted above, efficient HP killing of spores generally requires high temperatures, though these temperatures are significantly lower than needed to kill spores in the absence of concomitant HP treatment.

**Abrasion**

Treatment of spores with abrasives in either the wet or dry state can lead to spore disruption with or without prior spore germination (81, 82). Dormant spores are significantly more resistant to killing by abrasion than are growing cells or germinated spores. An intact spore
coat is not required for spore resistance to abrasion. Attempts to generate *B. subtilis* spores that completely lack an outer extremely insoluble protein layer termed the “rind” have been unsuccessful, so it remains possible that it is this structure that is responsible for spore abrasion resistance.

**Predation by bacteriovores**

*B. subtilis* spores are readily ingested by soil- and water-dwelling protozoa and nematodes, and wild-type *B. subtilis* spores are excreted by these organisms with their viability unchanged by their passage through digestive systems or phagocytic vacuoles (17, 18). In contrast, spores with coat defects that render the spores sensitive to lysozyme are rapidly digested by these bacteriovores, with the spore contents used to support the predators’ growth. However, not all spore components are digested, as the highly insoluble rind structure noted above is excreted. It has been suggested that the complex spore coat structure might have evolved in part to preclude spore destruction by predators that prey on bacteria.

**Freeze-thawing**

Spores are routinely resistant to multiple cycles of freezing and thawing, even in the absence of exogenous osmotic stabilizers, and even if the freezing process is extremely slow (11). In contrast, growing bacteria are often killed by multiple freeze-thaw cycles. This resistance property has not been well studied, although neither an intact spore coat nor α/β-type SASPs are involved in spore resistance to freezing and thawing.

**Desiccation**

Wild-type spores are resistant to multiple cycles of desiccation and rehydration, while growing bacteria are often killed by a single desiccation treatment unless specific compatible solutes are present in the solution from which the growing cells are dried (Table 1) (2, 11, 54). Two factors appear to play a role in spore desiccation resistance: (i) α/β-type SASPs and (ii) DPA (Table 1). In the absence of α/β-type SASPs, desiccation treatments do kill spores by DNA damage, and the viability of αβ− DPA-less spores is extremely low. Interestingly, while wild-type spores are essentially completely resistant to freeze-drying under low or high vacuums, desiccation under ultra-high vacuum (≤10⁻⁹ torr) does result in spore killing (79). This latter killing appears to be due to DNA damage, something also seen with growing cells. However, factors that might be involved in spore resistance to ultra-high vacuum have not been studied.

**Gas dynamic heating**

There has been significant interest recently in the killing of spores in a high-temperature gas environment at high shock pressures, undoubtedly because of interest in destroying *B. anthracis* spores being stored or developed as a potential bioweapon. Spores are indeed killed in such environments in milliseconds at temperatures >500 K (83, 84). However, the mechanism of spore killing in this type of environment has not been studied, nor have factors that help spores resist such treatment.

**Plasma**

There is currently significant interest in the use of nonthermal gas discharge plasmas for spore killing, in particular in decontamination of medical devices or packaged materials (85, 86). Unfortunately, there have been very few thorough studies of factors involved in spore resistance to or killing by nonthermal plasma. While a number of studies have noted severe morphological damage to spores by plasma, most of this damage seems likely to have taken place long after spore killing. There are a few studies indicating that plasma with significant associated UV radiation kills spores by DNA damage. However, plasma without associated UV radiation also can kill spores, suggesting there is a lethal target in addition to DNA, but this target has not been identified.

**Supercritical fluids**

Recent work has examined the use of supercritical fluids, in particular supercritical CO₂, for spore inactivation (14, 87, 88). While the use of supercritical CO₂ alone requires relatively high temperatures for spore killing, small amounts of additives such as H₂O₂ and water allow spore killing at more moderate temperatures and pressures. Spore killing by these supercritical fluids can take place with little obvious change in spore morphology or permeability. However, the mechanisms of spore killing by and resistance to supercritical fluids are not known.

**FINAL THOUGHTS**

While much is known about the mechanisms of spore resistance, there is still much to be learned. In spore resistance to wet heat, the precise mechanisms that modulate spore core water content remain unknown, as is the identity of specific proteins that are the targets of spore wet heat killing. In spore resistance to chemical biocides, the reason for the spore IM’s low permeability to such chemicals is also unknown, as is the precise likely IM damage that causes spore killing by many oxidizing agents. There are also a number of general questions...
that invite further work. Foremost among these is the question of whether mechanisms of spore resistance established largely with \textit{B. subtilis} spores are also the case with spores of other \textit{Bacillales} species as well as with spores of \textit{Clostridiaceae} species. While this seems likely, given the extreme applied importance of spores of a number of \textit{Clostridium} species, this would appear to be an important question to focus on, especially now that methods are available for genetic manipulation of a number of \textit{Clostridium} species.

Another question that has arisen recently concerns the heterogeneity in the resistance properties of individual spores in populations (8, 51, 52, 72, 74, 89). There is now significant evidence for heterogeneity in the resistance of spores to at least wet heat, and this seems likely to be true for resistance to other agents as well. What are the causes of this heterogeneity, and can the answer to this question give us further information on the mechanisms of spore resistance? There has been significant interest recently in heterogeneity in gene expression between individuals in bacterial populations, including sporulating cells, and this has been shown to play a major role in the heterogeneity in germination properties of spores in populations. While the causes of this heterogeneity in gene expression in sporulation are not completely understood, it seems likely to be in large part due to stochasticity. Might this also be the cause of heterogeneity in resistance properties of individual spores in populations?

Finally, it should be noted that there is increasing evidence that spore resistance is not completely static, but can change as spores “mature” (63, 75, 76). Thus, \textit{B. subtilis} spore wet heat resistance appears to increase markedly even after spores are released from the sporangium, although the precise mechanism for this effect is not known. In addition, there is recent evidence that there are significant changes in RNAs present in dormant spores, again well after their release from sporangia (90). While the precise meaning of the latter changes is unknown, it certainly seems possible that these changes could also modulate spores’ intrinsic resistance well after spores are released from sporangia. All in all, the resistance of spores undoubtedly still has a number of new and fascinating surprises in store for us.

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