REVIEW ARTICLE

Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals

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Abstract

A number of mechanisms are responsible for the resistance of spores of Bacillus species to heat, radiation and chemicals and for spore killing by these agents. Spore resistance to wet heat is determined largely by the water content of spore core, which is much lower than that in the growing cell protoplast. A lower core water content generally gives more wet heat-resistant spores. The level and type of spore core mineral ions and the intrinsic stability of total spore proteins also play a role in spore wet heat resistance, and the saturation of spore DNA with α/β-type small, acid-soluble spore proteins (SASP) protects DNA against wet heat damage. However, how wet heat kills spores is not clear, although it is not through DNA damage. The α/β-type SASP are also important in spore resistance to dry heat, as is DNA repair in spore outgrowth, as Bacillus subtilis spores are killed by dry heat via DNA damage. Both UV and γ-radiation also kill spores via DNA damage. The mechanism of spore resistance to γ-radiation is not well understood, although the α/β-type SASP are not involved. In contrast, spore UV resistance is due largely to an alteration in spore DNA photochemistry caused by the binding of α/β-type SASP to the DNA, and to a lesser extent to the photosensitizing action of the spore core’s large pool of dipicolinic acid. UV irradiation of spores at 254 nm does not generate the cyclobutane dimers (CPDs) and (6-4)-photoproducts (64PPs) formed between adjacent pyrimidines in growing cells, but rather a thymidyl-thymidine adduct termed spore photoproduct (SP). While SP is formed in spores with approximately the same quantum efficiency as that for generation of CPDs and 64PPs in growing cells, SP is repaired rapidly and efficiently in spore outgrowth by a number of repair systems, at least one of which is specific for SP. Some chemicals (e.g. nitrous acid, formaldehyde) again kill spores by DNA damage, while others, in particular oxidizing agents, appear to damage the spore’s inner membrane so that this membrane ruptures upon spore germination and outgrowth. There are also other agents such as glutaraldehyde for which the mechanism of spore killing is unclear. Factors important in spore chemical resistance vary with the chemical, but include: (i) the spore coat proteins that likely react with and detoxify chemical agents; (ii) the relative impermeability of the spore’s inner membrane that restricts access of exogenous chemicals to the spore core; (iii) the protection of spore DNA by its saturation with α/β-type SASP; and (iv) DNA repair for agents that kill spores via DNA damage.

Given the importance of the killing of spores of Bacillus species in the food and medical products industry, a deeper understanding of the mechanisms of spore resistance and killing may lead to improved methods for spore destruction.
Introduction
Spores of various Bacillus species are formed in sporulation, a process that is generally induced by reduced levels of nutrients in the environment (Driks 2002a; Piggot and Hilbert 2004). The formation of a spore generates a cell type that can survive for extended periods with little or no nutrients, yet is poised to return to life if nutrients become available. As spores may have to survive for long periods without nutrients, they are metabolically dormant, contain little or no high energy compounds such as ATP and NADH, exhibit no detectable metabolism of endogenous or exogenous compounds and little, if any, enzyme activity in the spore core, the analogue of the protoplast of a growing cell (Setlow 1983, 1994; Cowan et al. 2003). Because of their lack of enzyme action and metabolism, the dormant spore cannot repair damage to macromolecules such as DNA or protein. While there can be repair of DNA damage when the spore returns to life in the process of spore germination and outgrowth and metabolic activity resumes (Setlow 2003), if too much damage has been accumulated during spore dormancy, this damage can overwhelm the capacity of repair systems and lead to the death of the germinated spore (Setlow 1992, 1995; Setlow and Setlow 1996; Tennen et al. 2000). If spores were to stay in their dormant state for only a short period, perhaps DNA repair during spore outgrowth might be sufficient to ensure spore survival. However, spores can survive for many years, certainly hundreds, and there are reports suggesting that spores may survive for millions of years (Kennedy et al. 1994; Cano and Borucki 1995; Vreeland et al. 2000). Consequently, it is not surprising that spores have acquired a multitude of mechanisms to protect macromolecules from damage during their potentially long periods of dormancy. These mechanisms, which presumably evolved to prevent damage caused by long-term dormancy under moderate conditions, also protect spores from damage caused by acute treatments. It is this resistance to acute stress, as well as the ubiquity of spores in the environment that makes these organisms major agents of food spoilage and food-borne disease.

As is not surprising given the acute stresses to which spores are resistant, there are multiple mechanisms involved in spore resistance. These mechanisms appear to be conserved across Bacillus species, although most detailed studies on mechanisms of spore resistance have utilized spores of Bacillus subtilis because of the ease of molecular genetic manipulation of some strains of this species. Consequently, this review will concentrate on the resistance and killing of B. subtilis spores, although relevant work with other Bacillus species will also be cited. There has, unfortunately, been very little detailed work with spores of Clostridium species on their mechanisms of resistance to and killing by various stress treatments; this is a major gap in our knowledge, given the importance of the killing of spores of Clostridium species in foodstuffs and prepared foods.

Note that in considering spore killing by stress treatments it is essential to establish that the treated spores are indeed dead, and not just superdormant or only incapable of germinating under normal conditions. Thus a stress treatment may only inactivate some component of the spore’s germination apparatus and the treated spores may still be viable if they are germinated artificially. For example, spores treated with strong alkali appear dead when they are simply plated on rich media, but full viability can be restored by germination with exogenous lysozyme (Setlow et al. 2002). Consequently, when spore killing by a particular treatment is measured it is essential that: (i) the treated spores are plated on rich media to estimate viability, as treated spores sometimes appear dead when plated on poor media (Russell 1982; McDonnell and Russell 1999); and (ii) appropriate treatment with lysozyme is also tested for spore recovery.

While spores are resistant to a wide variety of treatments, this review will focus on spore resistance to radiation, heat and chemicals. For other reviews on spore resistance, the reader is referred to Russell (1982), Setlow (1992, 1994), McDonnell and Russell (1999), Nicholson et al. (2000), Setlow (2000) and Nicholson et al. (2005).

Spore structure and composition
The spore’s structure and chemical composition play major roles in spore resistance. Consequently discussion and understanding of spore resistance must start with a discussion of these features of spores. As is not surprising, the spore has a very different structure than that of a growing cell, with a number of features and constituents unique to spores (Fig. 1). Starting from the outside and proceeding inward the spore layers include the exosporium, coats, outer membrane, cortex, germ cell wall, inner membrane and central core.

The exosporium is a large loose-fitting structure found on spores of some species, in particular those of the Bacillus cereus group (Driks 2002b; Lai et al. 2003; Redmond et al. 2004; Waller et al. 2004). However, spores of many species, including B. subtilis, either do not contain an exosporium or if they do it is greatly reduced in size. As some proteins classified as coat proteins in B. subtilis are found in the exosporium of spores of Bacillus anthracis (Lai et al. 2003; Todd et al. 2003; Redmond et al. 2004), the exosporium may be an expanded version of the outermost coat layer. The exosporium is made up of proteins, including some glycoproteins found only in
Spore resistance

Figure 1 Spore structure. The various labelled spore layers are not drawn to scale, and the sizes of the various layers, in particular the exosporium, vary significantly between spores of different species.

Spores that contain an exosporium (Lai et al. 2003; Todd et al. 2003; Daubenspeck et al. 2004; Redmond et al. 2004). Although the function of these proteins and of the exosporium itself are not known, given the pathogenicity of many strains in the B. cereus group, this structure may be important in the spore’s interaction with target organisms.

The spore coat is a complex structure composed of several layers and contains ≥250 proteins in B. subtilis, most of which are spore-specific gene products (Driks 1999, 2002b; Lai et al. 2003). The function of most individual coat proteins is not known, although a few are morphogenetic proteins involved in overall spore coat assembly, as well as assembly of the exosporium (Bailey-Smith et al. 2005). The coat is important in spore resistance to some chemicals, to exogenous lytic enzymes that can degrade the spore cortex and to predation by protozoa, but has little or no role in spore resistance to heat, radiation and some other chemicals (Driks 1999; Nicholson et al. 2000; Setlow 2000; L.A. Klobutcher, K. Ragkousi and P. Setlow, unpublished data).

The precise function of the outer membrane that lies under the spore coats is not clear, although this membrane is an essential structure in spore formation (Piggot and Hilbert 2004). However, the outer membrane may not retain its integrity in dormant spores and consequently is likely not a significant permeability barrier. Indeed, removal of the outer membrane along with much spore coat protein has no notable effect on spore resistance to heat, radiation and some chemicals (Nicholson et al. 2000; Setlow 2000).

The cortex is composed of peptidoglycan (PG) with a structure similar to that of vegetative PG but with several spore-specific modifications (Popham 2002). The cortex is essential for formation of a dormant spore and for the reduction of the water content of the spore core. However, the mechanism whereby the spore cortex accomplishes this latter feat is not known. The cortex is degraded in spore germination, and this degradation is essential for spore core expansion and subsequent outgrowth (Setlow 2003). The germ cell wall lying under the cortex is also composed of PG, but with a structure probably identical to that of vegetative cell PG. The germ cell wall likely plays no role in spore resistance, but does become the cell wall of the outgrowing spore.

In contrast to the outer spore membrane, the inner spore membrane is a strong permeability barrier that plays a major role in spore resistance to many chemicals, in particular those that cross this membrane to damage spore DNA (Nicholson et al. 2000; Setlow 2000; Cortezzo and Setlow 2005). The inner membrane appears very compressed in the dormant spore, as the volume encompassed by this membrane increases approx. twofold in the first minutes of spore germination in the absence of ATP production (Cowan et al. 2004). Lipid molecules in the inner membrane are also largely immobile as determined by analysis of the fluorescence redistribution after photobleaching of lipid probes, but these probes become fully mobile upon spore germination (Cowan et al. 2004). The novel properties of the spore’s inner membrane are not due to an abnormal lipid composition, as the inner membrane’s lipid composition is very similar to that of the plasma membrane of growing cells (Cortezzo et al. 2004; Cortezzo and Setlow 2005).

The final spore layer is the core, the analogue of the growing cell’s protoplast. The core contains most spore enzymes as well as DNA, ribosomes and tRNAs. In almost all cases, the spore’s enzymes and nucleic acids are identical to those in growing cells, although there are some unique macromolecules in the core (see below) (Setlow 1983, 1994). There are also two small molecules whose levels in the core are important in spore resistance. The first is water. While water comprises 75–80% of the wet weight of the protoplast of a growing cell, water makes up only 27–55% of the spore core wet weight depending on the species (Gerhardt and Marquis 1989). The amount of free water in the spore core is also extremely low, such that macromolecular movement is greatly restricted (Cowan et al. 2003). The low core water content is likely the major factor in the spore’s enzymatic dormancy, and is the most important factor determining the spore’s resistance to wet heat (Gerhardt and Marquis 1989). However, the mechanism for the reduction in spore core water content during sporulation is not known. As expected, the spore core takes up water rapidly in the first minutes of spore germination and outgrowth when macromolecular motion and enzyme activity in the core are restored (Cowan et al. 2003; Setlow 2003).
The second core small molecule important in spore resistance is pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) (Fig. 2). This molecule comprises 5–15% of the dry weight of spores of both Bacillus and Clostridium species and is located only in the core, where it is most likely chelated with divalent cations, largely Ca$^{2+}$ (Gerhardt and Marquis 1989). DPA is synthesized in only the mother cell compartment of the sporulating cell, and is taken up into the forespore and is subsequently excreted in the first minutes of spore germination by mechanisms that are not well understood. The amount of DPA in the core is well above its solubility, whether chelated with divalent cations or not, but the precise state of the DPA in the core is not known. It has been suggested that the core has a glass-like state, but this has by no means been proved (Ablett et al. 1999; Leuschner and Lillford 2003). The accumulation of the huge depot of DPA in the spore core is responsible for some of the reduction in core water content during sporulation, and DPA also plays a significant role in the UV photochemistry of spore DNA (see below).

The third type of core molecule that plays an important role in spore resistance is a group of small, acid-soluble spore proteins (SASP) of the $\alpha/\beta$-type, so named for the two major proteins of this type in B. subtilis spores (Setlow 1992, 1995; Driks 2002b). The $\alpha/\beta$-type SASP are synthesized only in the developing forespore late in sporulation, slightly prior to DPA uptake. These small (60–75 aa) proteins are extremely abundant in spores, comprising 3–6% of total spore protein. The $\alpha/\beta$-type SASP are the products of a multi-gene family of from four to seven members in Bacillus species which are scattered around the genome. All members of this family appear to be expressed in parallel, generally with two proteins expressed at high levels and others at much lower levels. The amino acid sequences of these proteins are very highly conserved both within and across species, including Clostridium species. However, the $\alpha/\beta$-type SASP have no homologues in nonspore formers and exhibit no structural motifs identified in other proteins.

The $\alpha/\beta$-type SASP are found exclusively in the spore core where they saturate the spore DNA. The binding of these proteins alters DNA's structure and properties dramatically, and these proteins are significant factors in spore resistance to heat and many chemicals, and a major factor in spore resistance to UV radiation. Bacillus subtilis strains (termed $\alpha^+/\beta^-$) harbouring deletions of the genes encoding SASP-$\alpha$ and -$\beta$ lack approx. 80% of the $\alpha/\beta$-type SASP pool. The sporulation of $\alpha^+\beta^-$ strains is relatively although not completely normal, but $\alpha^+\beta^-$ spores exhibit reduced resistance to a variety of treatments, either because the slightly altered sporulation of the $\alpha^+\beta^-$ strain results in alteration in synthesis of spore coat proteins, or because the $\alpha/\beta$-type SASP protect spore DNA against damage (Setlow et al. 2000; Tennen et al. 2000; Genest et al. 2002; Young and Setlow 2003, 2004a,b; Shapiro et al. 2004). As expected the $\alpha/\beta$-type SASP are degraded early in spore outgrowth, and if not, their continued presence interferes significantly with subsequent development, presumably by blocking DNA transcription (Sanchez-Salas et al. 1992; Hayes and Setlow 2001).

**Radiation resistance**

Spores are significantly more resistant than are growing cells to ionizing ($\gamma$) radiation (Nicholson et al. 2000). The reasons for this elevated spore resistance are not all clear, but one factor may be the low core water content that presumably reduces the ability of $\gamma$-radiation to generate damaging hydroxyl radicals. However, this has not been proved, as there has been no systematic study of the relationship between core water content and the level of $\gamma$-radiation resistance. There is also no information on the possible role of DPA in spore resistance to $\gamma$-radiation, although DPA is involved in spore resistance to UV radiation (see below). However, the $\alpha/\beta$-type SASP are not important in spore $\gamma$-radiation resistance (Nicholson et al. 2000).

Spores are also 10- to 50-fold more resistant than are growing cells to UV radiation at 254 nm, the most efficient wavelength for spore killing (Nicholson et al. 2000, 2005). At longer wavelengths the difference in spore and growing cell resistance is generally smaller, although spores are still more resistant. One factor that may be involved in spore UV resistance is the shielding of UV-sensitive spore components (e.g. DNA) by UV-absorbing pigments located in the spore’s outer layers (Nicholson et al. 2005). However, such pigments are not present in spores of all Bacillus species.

The major reason for spore resistance to 254 nm radiation is the novel photochemistry of the DNA in spores. The major DNA products generated by 254 nm irradiation of growing cells are cyclobutane dimers (CPDs) and (6–4)-photoproducts (64PPs) (Fig. 3) formed between adjacent pyrimidines in the same DNA strand. However, very little of these latter two photoproducts are produced...
in wild-type spores (Table 1). By far the predominant photoproduct generated is a thymidyl-thymidine adduct termed the spore photoproduct (SP) (Fig. 3), also formed between adjacent thymidine residues on the same DNA strand. While intrastrand SP can be formed in DNA in vitro, only minute amounts of this photoproduct are formed in spores (Douki et al. 2005a). SP is generated in dormant spores by 254 nm radiation with a quantum efficiency that is similar to that for generation of CPDs and 64PPs in growing cells (Table 1). However, in spores SP is a much less lethal lesion than are CPDs or 64PPs, as SP is repaired in the first minutes of spore outgrowth by at least three repair mechanisms, one of which is specific for SP. This latter repair process uses an enzyme termed spore photoproduct lyase (Spl) that monomerizes SP to two thymidine residues. Spl is made only in the developing spore and is a member of the $S$-adenosylmethionine-dependent, radical-utilizing enzymes. In common with this group of enzymes, Spl contains an Fe-S cluster, likely [4Fe-4S], and appears to operate by a radical mechanism. A reaction mechanism involving an adenosyl radical has been proposed (Mehl and Begley 1999; Rebeil and Nicholson 2001; Cheek and Broderick 2002). In addition to Spl, SP is also repaired by recombination and excision repair, and both of the latter pathways (but not the Spl pathway) are dependent on RecA. Spores lacking either Spl or the RecA-dependent repair pathways are two- to sixfold more UV sensitive than are wild-type spores, and recA spl spores are approx. 50-fold more UV sensitive.

While efficient and rapid repair of SP is a major factor in spore UV resistance, the other factor is obviously the novel UV photochemistry of the DNA within spores. There are three factors that are involved in spore DNA photochemistry: (i) the saturation of spore DNA with $\alpha/\beta$-type SASP; (ii) the low water content in the spore core; and (iii) the high level of DPA in the spore core. The major factor is the binding of $\alpha/\beta$-type SASP to spore DNA, as both in vivo (Table 1) and in vitro $\alpha/\beta$-type

<table>
<thead>
<tr>
<th>Spore genotype</th>
<th>CPDs ($10^4$ bases per J cm$^{-2}$)</th>
<th>64PPs ($10^4$ bases per J cm$^{-2}$)</th>
<th>SP ($10^4$ bases per J cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1-0</td>
<td>0-1</td>
<td>252</td>
</tr>
<tr>
<td>$x/\beta$</td>
<td>102-9</td>
<td>78-9</td>
<td>108</td>
</tr>
<tr>
<td>DPA-less</td>
<td>8-7</td>
<td>12-2</td>
<td>23-3</td>
</tr>
<tr>
<td>$x/\beta$ DPA-less</td>
<td>108-1</td>
<td>95-3</td>
<td>4-1</td>
</tr>
</tbody>
</table>

*Spores of various strains were irradiated in water with UV that was predominantly at 254 nm, and the spore DNA was isolated, hydrolysed and photoproducts were determined by HPLC and mass spectrometry. The data are from Douki et al. (2005b).

†Values include levels of thymidine–thymidine, thymidine–cytosine and cytosine–thymidine CPDs. The level of the cytosine–cytosine CPD was <0-1 lesions per $10^4$ bases per J cm$^{-2}$.

‡Values include thymidine–thymidine and thymidine–cytosine 64PPs. Levels of cytosine–thymidine and cytosine–cytosine 64PPs were each <0-1 lesions per $10^4$ bases per J cm$^{-2}$.
SASP binding promotes SP formation and suppresses CPD and 64PP formation, likely because of some conformational change in DNA upon α/β-type SASP binding (Setlow 1992, 1995; Frenkiel-Krispin et al. 2004). However, the precise change in DNA structure in the α/β-type SASP-DNA complex is not clear.

Low DNA hydration levels have been implicated in causing SP formation in DNA in vitro (Rahn and Hosszu 1969; Patrick and Gray 1976), suggesting that the low core water content may also play a role in spore DNA photochemistry. However, there has been no systematic study of spore DNA photochemistry as a function of core hydration. DPA in the core also influences spore DNA photochemistry markedly, as DPA acts as a strong photosensitizer (Paidhungat et al. 2000; Douki et al. 2005b). Consequently, the DNA in DPA-less spores has significantly reduced photoreactivity relative to DPA-replete spores, whether or not the spores do or do not contain normal levels of α/β-type SASP.

Ultraviolet light at wavelengths longer than 254 nm will also kill spores, but is much less effective than 254 nm radiation. The DNA's photochemistry at longer wavelengths is also somewhat different than that at 254 nm (Nicholson et al. 2005). It appears likely that the factors important in determining spore DNA photochemistry at 254 nm are also important at longer wavelengths (Nicholson et al. 2005), but this topic needs further investigation. The photochemistry and photoreactivity of DNA also changes notably in spores that are UV irradiated in low or high vacuum (Nicholson et al. 2003, 2005), presumably because of subtle effects of the DNA hydration level on its structure and photochemistry.

**Heat resistance**

**Wet heat**

Probably the signature property of bacterial spores is their resistance to heat when suspended in an aqueous environment – termed wet heat resistance. Generally spores in water are resistant to approx. 40°C higher temperatures than are the growing cells of the same strain (Table 2) (for reviews see Gerhardt and Marquis 1989; Nicholson et al. 2000). The major factor that determines spore wet heat resistance is the core water content. With spores of different Bacillus species containing 27–55% of core wet weight as water, the lower the core water content the higher is the spore resistance to wet heat. For spores of single strains there is also an inverse relationship between core water content and wet heat resistance (Gerhardt and Marquis 1989; Melly et al. 2002b). The core water content of spores of a single species can be varied significantly in several ways. Spores formed at higher temperatures generally have lower core water contents than do spores formed at lower temperatures, with the latter spores having lower wet heat resistance than the former. Spores of strains that lack the ability to synthesize DPA can also be sporulated either with or without DPA in the medium (Paidhungat et al. 2000). The spores formed without DPA have much more core water than do the spores formed with DPA, as the latter have close to wild-type DPA levels. Presumably the extra core water in the DPA-less spores is removed when DPA is taken up into the core. As expected the DPA-less spores are much more sensitive to wet heat than are their DPA-replete counterparts.

While the major role of core water content in determining spore wet heat resistance is well established, how the low core water content protects spores from killing is less clear. Poorly hydrated proteins are generally more heat resistant than are proteins in solution, presumably because of reduced molecular motion, and proteins and DPA appear to be immobile in the spore core (Leuschner and Lillford 2000; Cowan et al. 2003). However, crucial knowledge that is lacking concerning spore core water is: (i) the precise level, if any, of free water in the core, and (ii) the degree of hydration of core proteins. It is also unclear how wet heat kills spores. Spore killing by wet heat is not via DNA damage, and is often accompanied by inactivation of core enzymes and rupture of the spore’s inner membrane permeability barrier (Warth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growing wild-type cells</th>
<th>Dormant spores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>recA</td>
</tr>
<tr>
<td>UV radiation (254 nm) (kJ m^-2)</td>
<td>36</td>
<td>330</td>
</tr>
<tr>
<td>Wet heat (90°C) (min)</td>
<td>&lt;0.1</td>
<td>18</td>
</tr>
<tr>
<td>Dry heat (120°C) (min)</td>
<td>–</td>
<td>18</td>
</tr>
<tr>
<td>Dry heat (90°C) (min)</td>
<td>5†</td>
<td>–</td>
</tr>
<tr>
<td>H2O2 (15% at 23°C) (min)</td>
<td>&lt;0.2</td>
<td>50</td>
</tr>
<tr>
<td>Formaldehyde (25 g l^-1) (min)</td>
<td>&lt;0.1</td>
<td>22</td>
</tr>
<tr>
<td>Nitrous acid (100 mmol l^-1) (min)</td>
<td>&lt;0.2</td>
<td>100</td>
</tr>
<tr>
<td>NaCl (50 mg l^-1, pH 7) (min)</td>
<td>&lt;0.1</td>
<td>5</td>
</tr>
<tr>
<td>Freeze dryings (number)</td>
<td>&lt;1</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

*Data are from experiments at 23°C unless noted otherwise, and are from Setlow (1992), Setlow and Setlow (1995, 1996), Loshon et al. (1999), Tennen et al. (2000) and Young and Setlow (2003).

Vegetative cells were dried in sucrose (Setlow and Setlow 1995).
1980; Setlow et al. 2002). However, the identity of the initial causal step in spore killing by wet heat has not been established.

Although the core water content is the major factor determining the level of spore wet heat resistance, the sporulation temperature, spore core mineralization and α/β-type SASP also contribute to spore wet heat resistance (Gerhardt and Marquis 1989; Nicholson et al. 2000; Setlow 2000). As mentioned above, spores of the same strain prepared at higher temperatures are more heat resistant than those made at lower temperatures. This is also true for spores of different species, as thermophiles invariably have more wet heat resistant spores than do mesophiles. Some of the higher wet heat resistance of spores of thermophiles is likely because of their relatively low core water content. However, the higher intrinsic thermostability of macromolecules, in particular proteins, from thermophiles also likely contributes to the increased wet heat resistance of their spores. In contrast to the situation in growing cells, heat shock proteins play no role in the wet heat resistance of spores (Melly and Setlow 2001).

In addition to high levels of DPA, the spore core also contains high levels of divalent cations, most chelated with DPA. This significant mineralization of the core is also important in spore wet heat resistance, with higher mineralization generally associated with higher spore wet heat resistance (Gerhardt and Marquis 1989). While some of the latter effect may be due to a decrease in core water content with increasing mineralization, the nature of the mineral ions in the core is also important. Although studies have not been extensive, available data indicate that spores with high Ca^{2+} levels are most wet heat resistant, spores with high levels of Mg^{2+} or Mn^{2+} are less heat resistant, and spores in which K^{+} or Na^{+} have been substituted for Ca^{2+} are the least wet heat resistant (Bender and Marquis 1985; Gerhardt and Marquis 1989). Other than affecting levels of core water, it is not clear why spore core mineralization and the specific nature of the mineral ions would affect spore wet heat resistance. Perhaps this is due to interaction of spore macromolecules with some DPA-metal ion lattice that permeates the spore core (Leuschner and Lillford 2000). Tied in with this latter uncertainty is whether DPA plays any direct role in spore wet heat resistance other than by reducing core water content.

The final factor important in spore wet heat resistance is the saturation of spore DNA with the α/β-type SASP (Setlow 1995, 2000; Nicholson et al. 2000). Wet heat does not kill wild-type spores by DNA damage. In contrast, spores lacking the majority of their α/β-type SASP (α^-β^- spores) are killed much more rapidly by wet heat than are wild-type spores and are killed by DNA damage, much of which is likely depurination (Table 2). The α/β-type SASP protect DNA in spores against a variety of types of damage, including depurination (Setlow 1995) and cytosine deamination (Sohail et al. 2002). Presumably in wild-type spores the DNA is so well protected by the binding of α/β-type SASP that spores are killed by some mechanism other than DNA damage. However, in α^-β^- spores the elevated temperatures to which wild-type spores are normally resistant cause potentially lethal DNA damage. As would be predicted based on the latter results, a recA mutation that eliminates much DNA repair in B. subtilis sensitizes α^-β^- spores to wet heat, while wild-type and recA spores have identical wet heat resistance (Setlow and Setlow 1996) (Table 2).

Dry heat

In addition to wet heat, spores are also significantly (c. 30°C) more resistant to dry heat than are the corresponding growing cells (Nicholson et al. 2000). Spores of thermophiles are no more dry heat resistant than are spores of mesophiles (Alderton and Snell 1969), but most factors potentially responsible for spore resistance to dry heat have not been investigated thoroughly, including DPA, core mineral content and sporulation temperature. However, the α/β-type SASP play a major role in the dry heat resistance of B. subtilis spores, as α^-β^- spores are as sensitive to dry heat as dry growing cells. The α/β-type SASP also protect against dry heat damage to DNA in vitro. Intrinsic to spore resistance to dry heat is also resistance to desiccation, and wild-type B. subtilis spores are resistant to multiple cycles of desiccation and rehydration (Table 2). To date the only factor that has been shown to contribute to spore desiccation resistance is the protection of spore DNA by the α/β-type SASP (Table 2).

In contrast to spore killing by wet heat, killing of wild-type spores by dry heat is accompanied by accumulation of DNA damage. The precise nature of this damage has not been determined but this damage is mutagenic and gives a mutation spectrum slightly different than that of wet heat treatment of α^-β^- spores (del Huesca-Espita et al. 2002). As expected since DNA damage accompanies spore killing by dry heat, DNA repair in spore outgrowth is also important in spore dry heat resistance. recA B. subtilis spores are much more sensitive to dry heat than are wild-type spores (Table 2), suggesting that RecA-dependent DNA repair systems are involved in the repair of dry heat damage to spore DNA. The genes for a number of DNA repair enzymes, including exoA, endoIV (ysfS) and the products of the ykoUVW operon are transcribed in the developing spore under the control of the forespore-specific sigma factor, σ^54, for RNA polymerase, and these enzymes are also involved in the repair of dry heat damage to DNA in wild-type spores (Weller et al. 2002; J.M.

**Chemical resistance**

Spores are extremely resistant to a variety of chemicals, including acids, bases, oxidizing agents, alkylating agents, aldehydes and organic solvents. As a consequence, spores are often the most resistant organisms that chemical decontaminants are designed to deal with – thankfully, spores are killed by some chemical treatments. In a few cases (e.g. formaldehyde, nitrous acid, alkylating agents) the mechanism of spore killing is via DNA damage, as the survivors accumulate mutations and a recA mutation sensitizes spores to these agents (Setlow et al. 1998; Loshon et al. 1999; Tennen et al. 2000) (Table 2). However, this is only true for a few genotoxic chemicals, and even oxidizing agents such as hydrogen peroxide that mutagenize growing cells do not do so to spores (Table 2).

Indeed, most oxidizing agents appear to kill spores by causing some type of damage to spores’ external layers, principally the spore’s inner membrane, such that when the treated spores germinate this damaged membrane ruptures resulting in spore death (Loshon et al. 2001; Genest et al. 2002; Melly et al. 2002a; Young and Setlow 2003, 2004a,b; Shapiro et al. 2004). Mildly lethal treatment of spores with a variety of oxidizing agents also sensitizes the survivors to a subsequent treatment (e.g. wet heat) to which an undamaged inner membrane may be required for full spore resistance (Cortezzo et al. 2004).

However, the precise nature of the inner membrane damage caused by oxidizing agents is not known, although it is not the oxidation of unsaturated fatty acids.

For some chemical agents including larger aldehydes such as glutaraldehyde and ortho-phthalaldehyde, the mechanism of spore killing remains unclear, although these latter two chemicals do not kill spores by DNA damage (Tennen et al. 2000; Cabrera-Martinez et al. 2002). Strong acid treatment appears to kill spores by causing them to ‘pop’ open, likely by rupturing the spore’s inner membrane permeability barrier in some fashion (Setlow et al. 2002). This may also be the mechanism for spore killing by organic solvents at elevated temperature. At least one treatment (strong alkali) thought to cause spore killing may not kill spores, as apparently alkali-killed spores can often be revived by appropriate treatment with lysozyme (Setlow et al. 2002). Alkali appears to inactivate the lytic enzymes needed for spore cortex hydrolysis in spore germination, as these enzymes are located in the spore’s outer layers where they are presumably more sensitive to alkali than are components located further within the spore.

In addition to different mechanisms of spore killing by different chemicals, there are also a number of mechanisms involved in spore resistance to different chemicals. The spore coat is of major importance in resistance to a large number of chemicals, in particular most oxidizing agents including chlorine dioxide, hypochlorite, ozone and peroxynitrite (Setlow 2000; Genest et al. 2002; Melly et al. 2002a; Young and Setlow 2003, 2004a,b), although the coat has only a minor role in spore resistance to hydrogen peroxide (Riesenberg and Nicholson 2000). No specific coat protein has been associated with spore resistance to these chemicals and the coat may be serving only as ‘reactive armor’, detoxifying these chemicals before they penetrate to more sensitive components further within the spore. At least one enzyme, superoxide dismutase, which might detoxify a potentially damaging chemical has been found associated with the exosporium and/or coat of spores of some species (Henriques et al. 1998; Lai et al. 2003; Redmond et al. 2004). However, it has not been demonstrated that this enzyme plays a role in spore resistance. Unlike the situation in growing cells where protoplast enzymes such as catalase, alkylhydroperoxidase reductase and superoxide dismutase play a major role in cell resistance by inactivating toxic agents, such enzymes play no role in dormant spore resistance, although they are present in the spore core (Casillas-Martinez and Setlow 1997).

A second important factor in spore chemical resistance is the spore’s inner membrane, which exhibits extremely low permeability to small hydrophilic and hydrophobic molecules (Gerhardt et al. 1972). Even a molecule as small as uncharged methylamine crosses this membrane extremely slowly; with *B. subtilis* the t1/2 for maximum methylamine uptake into the spore core is approx. 2 h at 23°C and even water may cross the spore’s inner membrane very slowly (Setlow and Setlow 1980; Seward et al. 1981; Westphal et al. 2003; Cortezzo et al. 2004; Cortezzo and Setlow 2005). An increase in the permeability of this membrane, whether achieved by sporulation at low temperatures or by mildly lethal treatment with any of a variety of oxidizing agents also leads to increased spore sensitivity to agents that kill spores by damaging spore DNA and thus must cross the spore’s inner membrane (Cortezzo and Setlow 2005). Unfortunately, while the low permeability of the spore’s inner membrane is clearly an important factor in resistance to some chemicals, the reason for this membrane’s low permeability is not known, although this seems likely to be related to the immobility of inner membrane lipids noted above.

The third factor important in spore resistance to some chemicals is the saturation of spore DNA with α/β-type SASP (Nicholson et al. 2000; Setlow 2000). Thus
wild-type spores are not killed by hydrogen peroxide via DNA damage, while \(\alpha^-\beta^-\) spores are much more sensitive to this agent and are killed via DNA damage (Table 2). The \(\alpha/\beta^-\) type SASP also protect DNA in vitro against damage by hydrogen peroxide. For both formaldehyde and nitrous acid, even wild-type spores are killed via DNA damage, but \(\alpha^-\beta^-\) spores are much more sensitive to these agents (Loshon et al. 1999; Tennen et al. 2000) (Table 2). However, the \(\alpha/\beta^-\) type SASP do not protect spore DNA against all chemicals, as alkylating agents such as ethylmethanesulfonate kill wild-type and \(\alpha^-\beta^-\) spores at the same rate, and the \(\alpha/\beta^-\) type SASP do not block DNA alkylation by this type of agent in vitro (Setlow et al. 1998).

**Summary and future considerations**

It is perhaps not surprising given the multiplicity of stress treatments to which spores of Bacillus species are resistant that there are multiple mechanisms that protect spore against stress (Table 3). Indeed, even for individual stresses there are multiple factors giving rise to spore resistance. Major factors involved in spore resistance to heat, radiation, and chemicals include (Table 3): (i) the coats, providing a barrier against lytic enzymes, toxic chemicals and perhaps UV radiation; (ii) the inner membrane, whose low permeability retards influx of toxic chemicals into the core; (iii) the core’s low water content, crucial in protection against wet heat and perhaps \(\gamma^-\) radiation, and likely an important factor in DNA photochemistry and thus UV resistance; (iv) the saturation of spore DNA by \(\alpha/\beta^-\) type SASP protecting against wet and dry heat and some genotoxic chemicals, as well as changing the DNA’s photochemistry leading to UV resistance; (v) core mineralization, likely a factor in resistance to wet heat; (vi) core DPA, influencing core hydration and mineral levels and thus wet heat resistance, and also DNA’s UV photochemistry; (vii) the intrinsic thermostability of a strain’s proteins, contributing to wet heat resistance; and (viii) DNA repair, important in resistance to DNA damaging treatments such as radiation, dry heat and genotoxic chemicals.

While we have learned much about spore resistance to and killing by heat, radiation, and chemicals in recent years, there are still a number of major gaps in our knowledge. Among the more notable of these gaps are the following:

1. Despite that tremendous importance of spore resistance to and killing by heat, radiation and chemicals, we still lack detailed understanding of the mechanisms of both of these processes. It is clear that the core’s low water content is the major factor in spore wet heat resistance, but we do not know: (i) how this low water content is achieved; (ii) the level of free water, if any, in the core; (iii) the degree of hydration of core proteins; and (iv) how a low core water content provides wet heat resistance. We also do not know how wet heat kills spores, although we do know some ways (e.g. DNA damage) whereby wet heat does not kill spores.

2. While we know how some chemicals commonly used as disinfectants (e.g. formaldehyde) kill spores, for others, in particular oxidizing agents, we do not. We suspect that the latter agents cause some type of damage to the spore’s inner membrane such that this damage causes membrane rupture and cell death upon spore germination and outgrowth. However, we do not know the precise nature of the membrane damage caused by oxidizing agents. This information could be extremely valuable, as it might indicate why spores are resistant to such agents, and how such resistance might be lowered or better dealt with.

3. It seems clear that the relatively low permeability of the inner membrane is important in spore resistance to many chemicals. However, we know much too little about the precise structure of the inner membrane of the dormant spore, other than that this structure is most likely significantly different than that of the plasma membrane of an outgrowing spore or a growing cell. As the spore’s inner membrane is: (i) the major barrier preventing the loss of small molecules such as DPA that are essential for spore resistance; (ii) the location of a number of proteins

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**Table 3 Mechanisms of spore resistance to and killing by heat, radiation and chemicals**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Factors in spore resistance</th>
<th>Mechanism of spore killing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet heat</td>
<td>Low core water; (\alpha/\beta^-) type SASP; core mineralization; DPA; (\alpha^-\beta^-)</td>
<td>? (not DNA damage)</td>
</tr>
<tr>
<td></td>
<td>thermostability of a strain’s proteins</td>
<td>DNA damage</td>
</tr>
<tr>
<td>Dry heat</td>
<td>(\alpha/\beta^-) type SASP; DNA repair</td>
<td>DNA damage</td>
</tr>
<tr>
<td>UV radiation</td>
<td>DNA photochemistry; DPA; (\alpha/\beta^-) type SASP; low core water; DNA repair</td>
<td>DNA damage</td>
</tr>
<tr>
<td>(\gamma^-) Radiation</td>
<td>?</td>
<td>DNA damage</td>
</tr>
<tr>
<td>Chemicals</td>
<td>Coats; inner membrane impermeability; (\alpha/\beta^-) type SASP; DNA repair</td>
<td>DNA damage (some); inner membrane damage (some); ? (some)</td>
</tr>
</tbody>
</table>
involved in the triggering of spore germination; and (iii) where membrane active agents such as cationic surfactants act to trigger spore germination (Setlow et al. 2003), it seems likely that detailed knowledge of the structure of the inner membrane will give us new insight into mechanisms not only of spore resistance, but also spore dormancy and spore germination.

(4) We currently know next to nothing about the mechanism of spore resistance to γ-radiation. The experiments to gather this knowledge are all relatively straightforward, and only require that they be done. As γ-radiation is being used with greater and greater frequency for sterilization of materials from food to mail, it would be most worthwhile to determine the mechanisms whereby spores resist such treatment.

In looking at the overall situation with regard to our knowledge of the mechanisms of spore resistance to and killing by heat, radiation and chemicals, it should be obvious that there is still much to be learned. Indeed, some of the major questions that were first raised ≥50 years ago, in particular on the mechanism of spore killing by and resistance to wet heat, have still not been answered. Clearly, there is yet much work to be done.

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References


