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I will survive: DNA protection in bacterial spores

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Dormant spores of *Bacillus*, *Clostridium* and related species can survive for years, largely because spore DNA is well protected against damage by many different agents. This DNA protection is partly a result of the high level of Ca^{2+}-dipicolinic acid in spores and DNA repair during spore outgrowth, but is primarily caused by the saturation of spore DNA with a group of small, acid-soluble spore proteins (SASP), which are synthesized in the developing spore and then degraded after completion of spore germination. The structure of both DNA and SASP alters upon their association and this causes major changes in the chemical and photochemical reactivity of DNA.

Spore DNA resistance

Dormant spores of *Bacillus* and *Clostridium* species and their close relatives are formed in sporulation (Box 1; Box 2; Box 3) and can survive for hundreds of years and perhaps longer [1–3]. The physiological properties of these spores are different from those of other known cells – *Bacillus* and *Clostridium* spores in water exhibit no detectable metabolism of endogenous or exogenous compounds and can survive treatments with wet and dry heat, UV and γ-radiation, desiccation and toxic chemicals that rapidly kill growing bacteria [4–10] (Table 1). Although many factors contribute to spore resistance and long-term survival, minimization of damage to spore DNA is essential. Two mechanisms that minimize spore DNA damage are protection and repair. The former option should be preferred for spores because DNA repair can be mutagenic and repair of dormant spore DNA damage must wait until spore germination is complete and spore outgrowth begins (Box 4). As far as is known, spores contain only a single chromosome, thus precluding homologous recombination early in outgrowth as a mechanism to repair damage in dormant spore DNA, although there are several spore-specific DNA repair proteins [4–7,11,12]. Whereas DNA repair contributes to spore DNA resistance to some treatments, protection of DNA is the major factor in spore DNA resistance [4–7].

Spores of *Bacillus* and *Clostridium* species have long been of interest because of their novel properties and the importance of these spores as agents of food spoilage and food-borne disease. However, interest in spores has grown in recent years due to the potential use and, more recently, the actual use of *Bacillus anthracis* spores as a bioterrorism weapon. Because spore resistance – in particular the resistance of spore DNA – is a major factor in the ability of spores to cause disease, it is thus timely to review mechanisms of DNA protection in spores, especially because this topic has not been reviewed in detail in several years and there have been some important recent advances. This review will first describe general features of spore structures and constituents that do or do not contribute to spore DNA resistance, and then discuss in detail the major factor that protects spore DNA from damage: the α/β-type small, acid-soluble spore proteins (SASP) that saturate DNA in the dormant spore. The extreme protection given by the binding of these novel proteins to spore DNA is unique in biology.

DNA protection in spores

The spore DNA is located in the core (Box 2), a region that has a lower water content (25–50% wet weight) than the protoplast of a growing cell or the outer spore layers (~80% of wet weight is water in both) [13]. The spore core also has a pH of ~6.5, which is ~1 pH unit lower than in growing cells, and has a huge amount of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA); ~20% of core dry weight] [13,14] (Box 1, Box 2). DPA in spores is almost certainly present as a 1:1 chelate with divalent cations, predominantly Ca^{2+}, giving Ca-DPA. Another unique feature of the core is the saturation of spore DNA with α/β-type SASP, named after the major proteins of this type in *Bacillus subtilis* spores [5,14–16]. The α/β-type SASP are synthesized in developing spores late in sporulation, and are degraded early in spore outgrowth.

Any or all of the unique features of the spore core could be important for protection of spore DNA. However, neither the low core water content of the spore nor its slightly low pH are important in DNA protection because alterations in these factors do not appreciably alter spore DNA resistance. Ca-DPA does contribute to spore DNA resistance to wet and dry heat, desiccation and hydrogen peroxide, but actually sensitizes DNA in spores to UV radiation [17,18].

The core component that gives spore DNA most protection is the α/β-type SASP. There are high levels (5–10% of total core protein) of these proteins in spores of *Bacillus* and *Clostridium* species and their close relatives, and in *Bacillus megaterium* and *B. subtilis* spores (and possibly other species) there is sufficient α/β-type SASP to saturate spore DNA [5,7,14–16]. Binding of these proteins to DNA in spores has been shown by

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Box 1. Sporulation

Sporulation of *Bacillus* species [51] is induced by starvation for carbon and/or nitrogen. This process is initiated by phosphorylation of the Spo0A protein, which is both an activator and repressor of gene expression. A major function of Spo0A-phosphate is to activate expression of genes encoding two sigma (σ) factors that associate with RNA polymerase and determine the specificity of this enzyme. As sporulation proceeds, there is an unequal cell division that generates a large mother cell and a smaller forespore. The plasma membrane of the mother cell then grows around the forespore, generating an engulfed forespore surrounded by two apposed membranes. Activation and/or synthesis of additional σ factors in a compartment-specific manner then drives different patterns of gene expression in the mother cell and forespore compartments, with σ^F and σ^G driving forespore gene expression [12] and σ^E and σ^K driving mother cell gene expression. There is extensive 'cross-talk' between the two compartments of the sporulating cell, in particular through developmental checkpoints, ensuring that gene expression in both compartments remains temporally coordinated. The subsequent synthesis of a thick peptidoglycan cortex between the outer and inner forespore membranes is accompanied by a large decrease in the volume and water content of the forespore protoplast, and there is also a decrease in forespore pH. The forespore later takes up tremendous amounts of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] that has been synthesized in the mother cell, which decreases forespore protoplast water content even further. Throughout late stages of sporulation a complex proteinaceous coat is layered on the outer surface of the spore using coat proteins synthesized in the mother cell; in some species a large external balloon-like layer termed the exosporium is added as well. Finally the mother cell lyses, releasing the spore into the environment.

cross-linking and immunoelectron and immunofluorescence microscopy. These proteins are crucial for spore DNA protection, as shown by the sensitivity of DNA to many DNA-damaging agents in *B. subtilis* spores that lack ~85% of their α/β-type SASP (termed $\alpha^-\beta^-$ spores), and the protection of DNA *in vitro* against damage upon binding of α/β-type SASP. Decreased levels of α/β-type SASP also decrease the resistance of *Clostridium perfringens* spores to wet heat and UV radiation [19,20]. α/β-type SASP and Ca-DPA together are also essential for spore DNA survival because spores that lack both of these components rapidly lose viability during sporulation owing to DNA damage [17].

α/β-type SASP

The α/β-type SASP are encoded by monocistronic genes, termed *ssp*, which are members of a multigene family of 3–7 members in spore-formers (Figure 1; Figure 2). The *ssp* genes are scattered on chromosomes (and on a plasmid in *Bacillus cereus* ATCC 10987; Figure 1), their promoters are upstream of and close to a strong ribosome-binding site, and coding sequences are followed by potential stem-loop structures that are probably transcription stop signals. Proteins encoded by *ssp* genes are not processed, except for removal of the N-terminal methionine. Because some species have so many *ssp* genes, one or more might be pseudogenes. Indeed, one *ssp* pseudogene is present in all strains of *B. anthracis* that have been sequenced and

Box 2. Spore structure

The structure of dormant spores of *Bacillus* species [51] (Figure I) is very different from that of growing cells. On the outside is the exosporium, a balloon-like structure composed of proteins and carbohydrate found on spores of some species (e.g. *Bacillus anthracis*) but not others (e.g. *Bacillus subtilis*). The function of the exosporium is unknown, although it might be important in pathogenesis by spores. The underlying proteinaceous coat layer contains >40 different proteins, almost all being spore-specific [52]. The coat protects spores against predatory eukaryotic microbes and reactive chemicals [4,5,53]. The outer spore membrane is just below the coat and is essential in spore formation but might not be a permeability barrier in mature spores. The cortex beneath the outer membrane is composed predominantly of peptidoglycan (PG) with a structure similar to that of PG in growing cells. However, spore cortex PG has two modifications: muramic acid-δ-lactam (MAL) and muramic acid linked only to alanine – these are not present in growing cell PG. The cortex and the underlying germ cell wall are essential for maintenance of the integrity of the spore inner membrane. The germ cell wall is also composed of PG whose structure is identical to that of growing cell PG, and becomes the cell wall of the outgrowing spore. The inner membrane has low permeability to small molecules, perhaps even water [54,55]. Lipid probes are largely immobile in this membrane, which seems to be significantly 'compressed' [56] (Box 4). The low permeability of this membrane is one factor in the resistance of spores to some chemicals, in particular DNA-damaging chemicals [4,5,55]. The final layer is the core that contains the spore DNA, RNA and most enzymes. The low water content in the core (25–50% of wet weight depending on the species), the high level of Ca-DPA (25% of core dry weight) and the saturation of DNA with α/β-type small, acid-soluble spore proteins all contribute to spore resistance properties [4–10,13–17].

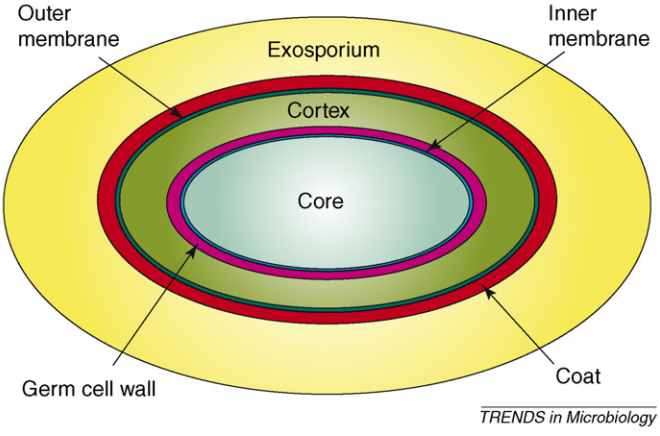

Figure I. Structure of a bacterial spore. The relative sizes of various layers are not drawn to scale, and the large exosporium is not present in spores of all species. Note that there can be several sublayers in individual layers, in particular the coat and probably the exosporium.

Box 3. Spore properties

Spores of *Bacillus* and *Clostridium* species are dormant, and contain little if any common high-energy compounds such as nucleoside triphosphates [14,51]. Even more notable is that spores contain significant levels of a variety of metabolites [e.g. 3-phosphoglyceric acid (3PGA)] and enzymes for the catabolism of these compounds, yet exhibit no metabolic activity. Reasons for the establishment of spore dormancy include the decrease in forespore pH (Box 1) that regulates 3PGA catabolism. However, the major factor causing and maintaining spore dormancy is the low water content in the spore core where metabolites and metabolic enzymes are located [13]. Levels of water here are so low that at least one normally mobile protein is immobile [57]. Presumably, levels of core water are too low to allow enzyme action.

The other defining property of spores is their resistance [4–10,13–15,51]. The hallmark spore resistance is to wet heat: spores in water commonly resist temperatures ≥40 °C higher than do growing cells. Indeed, spores are so resistant to moist heat that spores of *Bacillus stearothermophilus*, a thermophile, are used to test autoclave function. Spores are also resistant to many other treatments including dry heat, desiccation, UV and γ-radiation, high pressure, mechanical disruption and many toxic chemicals including acids, alkalis, alcohols, aldehydes, oxidizing agents and a variety of DNA-damaging agents. The extreme resistance of spores to these treatments, their presence throughout the environment, and the ability of growing cells of some spore formers to spoil food, synthesize dangerous toxins or cause human disease (including anthrax) has made the development of regimens and reagents for killing spores of continued interest [51].

Box 4. Spore germination and outgrowth

Although spores can remain dormant for years, addition of specific nutrients can trigger the rapid 'return to life' through spore germination followed by outgrowth [51,58]. Nutrients that trigger germination are specific for the strain or species of the spore former, with common nutrient germinants being L-amino acids, D-sugars and purine nucleosides, but metabolism of nutrient germinants is not required for germination. Rather, the nutrients bind to proteins in the spore inner membrane termed germinant receptors, which are found only in spores, and this binding triggers the release of monovalent cations (H^+, K^+) and DPA plus its divalent cations. How ion release is triggered and takes place is not known. The release of these ions is balanced by water uptake but the resultant increase in core water content is not sufficient to break spore dormancy or allow protein movement [57], although spore wet heat resistance decreases. These latter events encompass stage one of germination. These events then trigger the hydrolysis of cortex PG, which in *Bacillus subtilis* spores is catalyzed by two redundant enzymes, CwlJ and SleB, which recognize MAL in PG substrates. CwlJ is probably activated directly by released Ca-DPA but the mechanism of SleB activation is unknown. Cortex PG hydrolysis then enables the spore core to expand (although without any membrane synthesis), raising the core water content to that of a growing cell and completing germination, which takes place without any ATP production. Completion of germination then allows protein mobility [57] and enzyme action in the core and leads to spore outgrowth. Key events in outgrowth include SASP hydrolysis, metabolism of exogenous and endogenous compounds and macromolecular synthesis, culminating in DNA replication.

annotated (see legend to Figure 1), and other possible *ssp* pseudogenes exist (see Figure 1 for an example of a *Bacillus thuringiensis ssp* gene that has no ribosome-binding site).

The α/β-type SASP from the aerobic spore-formers have 59–75 amino acids, not counting the N-terminal methionine (Figure 1), with more size variation among the proteins from the anaerobes (Figure 2). The difference in size between different proteins is largely in the length of the N- and C-terminal regions, which also exhibit the greatest sequence variation. In aerobic spore-formers, the sequences of central regions of α/β-type SASP are extremely similar, both within and across species (Figure 1). Sequences of the proteins from anaerobic spore-formers also exhibit a central region of homology, although this is less similar than in the aerobes (Figure 2). However, there are clear regions of sequence homology between α/β-type SASP in the aerobic and anaerobic spore-formers (compare highlighted regions in Figure 1 and Figure 2).

Although α/β-type SASP sequences are highly conserved, these sequences show minimal homology to sequences in current databases and contain no known sequence motifs, including motifs found in DNA-binding proteins. However, the regions highlighted in red in Figure 1 and Figure 2 surround the site where these proteins are cleaved by a specific protease early in spore outgrowth (see later) to free up spore DNA for transcription. A second, more highly conserved region encompasses 12–19 amino acids (Figure 1 and Figure 2; highlighted in yellow) and mutation of at least one of these residues (conversion of the glycine residue in green in Figure 1 to alanine) greatly reduces DNA binding by the variant protein both *in vitro* and *in vivo*. By contrast, the residues in the protease cleavage site exhibit several differences

Table 1. Resistance of growing *Bacillus subtilis* cells and spores to various agents[a,b]

Agent[c]	Growing cells	Dormant spores			
	Wild-type	Wild-type	*recA*	$\alpha^-\beta^-$	$\alpha^-\beta^-$ *recA*
Wet heat (90°) (min)	<0.1	18	25	2.5	≤0.5
Wet heat (75 °C) (min)	–	–	–	100	7
Dry heat (120 °C) (min)	–	18	2	–	–
Dry heat (90 °C) (min)	5[d]	–	–	2	≤0.2
Freeze dryings (number)	<1	>20	>20	3	1
H_2O_2 (15%) (min)	<0.2	50	55	15	3
Formaldehyde (15 g/L)[e] (min)	≤0.1	28	12	–	–
Formaldehyde (10 g/L)[e] (min)	–	–	–	11	4
Nitrous acid (100 mM) (min)	≤0.2	110	18	7	≤1
UV radiation at 254 nm (kJ/m^2)	36	330	185	18	3

[a]Data are from experiments with growing cells and dormant spores of *B. subtilis* at 23 °C unless noted otherwise, and are from Refs [5] and [8–10].
[b]Dash (–) indicates data not available.
[c]Units represent time (min), UV dose (kJ/m^2) or numbers of freeze-dryings required to kill 90% of the population.
[d]Growing cells were dried with sucrose.
[e]Treatment was at 30 °C.

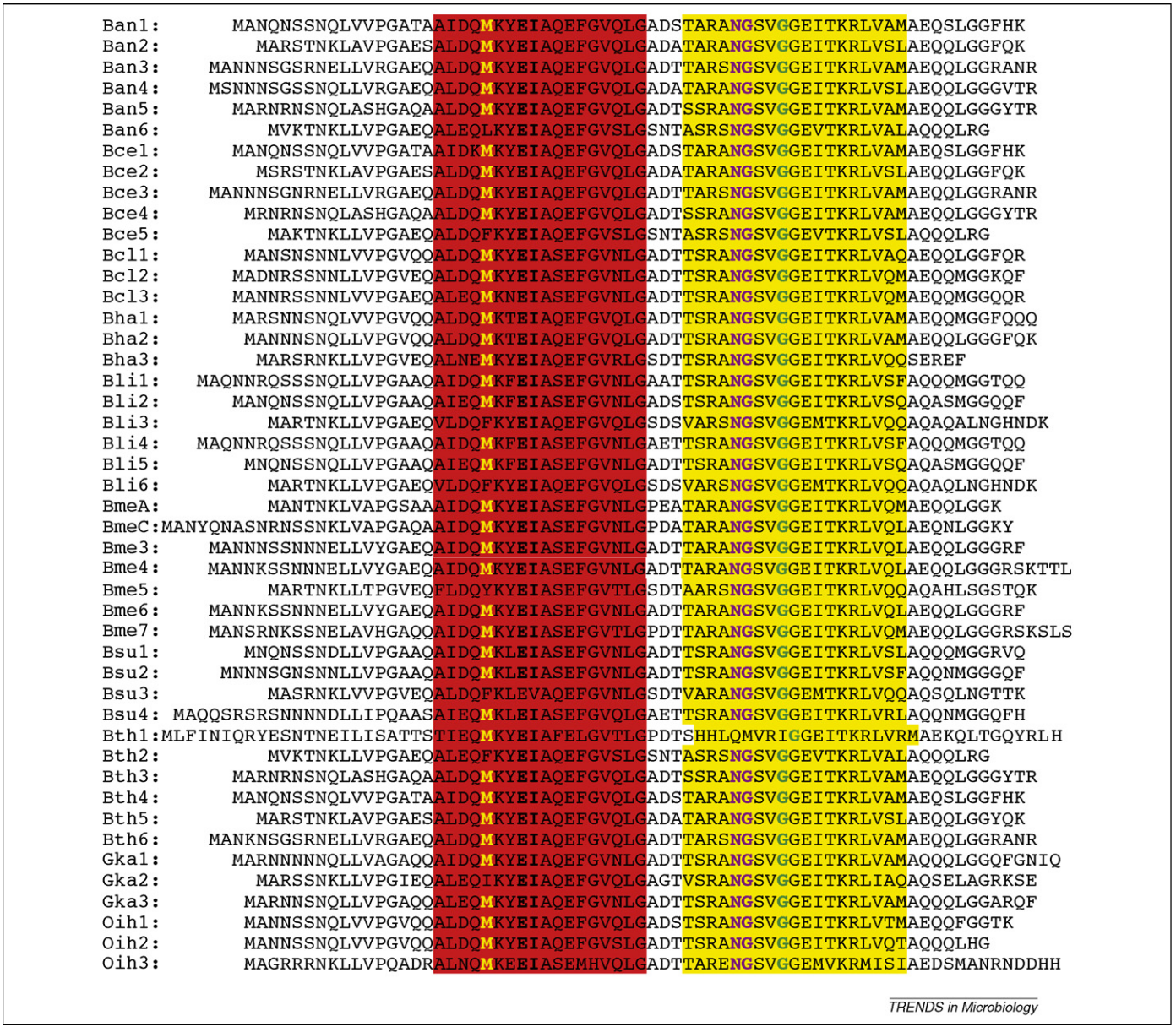

Figure 1. Amino acid sequences of all α/β-type SASP from species in the aerobic line of spore formers. The sequences are given in the one letter amino acid code and are from the one species [*Bacillus megaterium* (Bme)] in which all *ssp* genes have been individually cloned and sequenced, or from species whose genome has been completely sequenced and annotated in the NCBI database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=microb). The highly conserved region highlighted in red surrounds the single site in these proteins cleaved by the germination protease Gpr, with the cleavage site between the Glu-Ile or Glu-Val sequences emboldened in black. A second highly conserved region that might be involved in DNA binding is highlighted in yellow. A relatively well conserved Met residue at which oxidation is prevented by DNA binding and where oxidation greatly decreases DNA binding is emboldened in yellow; a conserved Asp-Gly sequence at which Asp deamidation is prevented by DNA binding and where deamidation decreases DNA binding is emboldened in purple; a conserved Gly residue whose change to Ala greatly decreases DNA binding *in vitro* and *in vivo* is emboldened in green. The species from which these sequences have been obtained all group with Bacillales (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=microb) and are: Ban, *Bacillus anthracis* Ames; Bce, *Bacillus cereus* ATCC 10987; Bcl, *Bacillus clausii* KSM-K16; Bha, *Bacillus halodurans* C-125; Bli, *Bacillus licheniformis* ATCC 14580; Bme, *B. megaterium* QM B1551; Bsu, *Bacillus subtilis* subspecies subtilis strain 168; Bth, *Bacillus thuringiensis* serovar konkukian strain 97–27; Gka, *Geobacillus kaustophilus* HTA426; and Oih, *Oceanobacillus iheyensis* HTE831. All genes shown have strong ribosome-binding sites (RBS) just upstream of the translation initiation codons except for Bth1 (nucleotides 2966754 → 296984 in the genome of *B. thuringiensis* serovar konkukian strain. 97–27) in which no RBS is present (P. Setlow, unpublished). This gene might thus be a pseudogene, an assignment that is consistent with the significant divergence of the sequence of the encoded protein from those of the other α/β-type SASP in this figure. There is also a likely pseudogene in all sequenced *B. anthracis* strains (nucleotides 3034917 → 3035131 in *B. anthracis* strain Ames; not shown in the figure) that also has no RBS and a frame-shifting mutation in its coding sequence (P. Setlow, unpublished). All genes shown are on the chromosome but in *B. cereus* strain E33L (not shown in the figure), one *ssp* gene is on the large plasmid pE33L466 of this strain (P. Setlow, unpublished).

across species (Figure 1; Figure 2) and can be changed with little to minimal effects on DNA binding [21].

In spore-formers that have been studied, all *ssp* genes are expressed in parallel only in the developing spore and under the control of the forespore-specific σ factor σ^G. However, in *Bacillus* species, two *ssp* genes are generally expressed at high levels whereas others are expressed at much lower levels, and when *ssp* mRNAs are being translated, the α/β-type SASP comprise ~50% of all protein made in the developing spore. Because the α/β-type SASP are DNA binding proteins, their synthesis might have general effects on transcription. Indeed, the absence of synthesis of major α/β-type SASP in *B. subtilis* causes global changes in gene expression in the developing spore and mother cell [22].

Figure 2. Amino acid sequences of all α/β-type SASP from species in the anaerobic line of spore formers. The sequences are given in the one letter code and are from species whose genomes have been completely sequenced and annotated in the NCBI database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=microb). The moderately well conserved region highlighted in red surrounds the single site in these proteins cleaved by Gpr, with the cleavage site between the two emboldened residues; this site of Gpr cleavage is inferred from that in proteins of *Bacillus* species. A second well conserved region that might be involved in DNA binding is highlighted in yellow. The species from which the sequences have been obtained group with Clostridia (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=microb) and are: Chy, *Carboxydothermus hydrogenoformans* Z-2901; Cac, *Clostridium acetobutylicum* ATCC 824; Cno, *Clostridium novyi* NT; Cpe, *Clostridium perfringens* SM101; Cte, *Clostridium tetani* E88; Dha, *Desulfitobacterium hafniense* Y-51; Mth, *Moorella thermoacetica* ATCC 39073; Swo, *Syntrophomonas wolfei* subspecies wolfei strain Goettigen; and Tte, *Thermoanaerobacter tengcongensis* MB4.

There are other SASP present in spores besides those of the α/β-type. These additional SASP have little sequence homology to the α/β-type SASP and include the single γ-type SASP that is the most abundant SASP in spores of aerobes (but not present in anaerobes), and a large number of less abundant SASP [14–16,23]. In spores of *Bacillus* species some of the less abundant SASP are of the α/β-type; others are also spore-specific and most of these are made in parallel with α/β-type SASP. However, no function is known for these additional SASP, other than for SASP-γ, the degradation of which (along with that of α/β-type SASP) supplies amino acids for the outgrowing spore [14–16]. However, less abundant α/β-type SASP can substitute for their more abundant brethren in some situations (see later).

Effects of α/β-type SASP on DNA properties in spores

Studies *in vivo* and *in vitro* have shown that α/β-type SASP have profound effects on DNA properties. Studies *in vivo* have largely used wild-type and $\alpha^-\beta^-$ *B. subtilis* spores. The $\alpha^-\beta^-$ spores are significantly more sensitive than wild-type spores to wet and dry heat, UV radiation, dessication and genotoxic chemicals including nitrous acid, hydrogen peroxide and formaldehyde, although $\alpha^-\beta^-$ and wild-type spores exhibit similar resistance to γ-radiation and DNA alkylating agents [4,5,9,10,24] (Table 1). The resistance of $\alpha^-\beta^-$ spores is restored by synthesis of DNA-saturating amounts of several α/β-type SASP, including normally very abundant and less abundant proteins. This suggests that all wild-type α/β-type SASP have similar effects on DNA properties (see later). It is particularly notable that several agents (wet heat, hydrogen peroxide, desiccation) do not kill wild-type spores by DNA damage as shown by the lack of: (a) detectable DNA damage associated with spore killing; (b) mutations in survivors; and (c) effect of loss of several DNA repair proteins on spore resistance to these agents [4–7,11,12] (Table 1). By contrast, wet heat, desiccation and hydrogen peroxide do kill $\alpha^-\beta^-$ spores by DNA damage as shown by: (a) the presence of detectable DNA damage accompanying $\alpha^-\beta^-$ spore killing; (b) high levels of mutations in survivors; and (c) large decreases in $\alpha^-\beta^-$ spore resistance upon loss of various DNA repair proteins [4–7,11,12] (Table 1). Other agents (formaldehyde, nitrous acid, dry heat, UV radiation) kill both wild type and $\alpha^-\beta^-$ spores by DNA damage, although $\alpha^-\beta^-$ spores are killed more readily [4–12] (Table 1). As noted earlier, when spore killing is by DNA damage, DNA repair during spore outgrowth (in part by RecA-dependent processes) is an important factor in spore resistance [4–12] (Table 1).

Except for wet heat and UV radiation, the type of DNA damage that kills wild-type or $\alpha^-\beta^-$ spores is not known, although the DNA damage caused by dry heat in wild-type spores and desiccation and hydrogen peroxide in $\alpha^-\beta^-$ spores is different than that caused by wet heat in $\alpha^-\beta^-$ spores [25]. Dry heat also probably causes at least some double-strand breaks in spore DNA because proteins homologous to eukaryotic enzymes involved in non-homologous end joining of DNA are important in spore

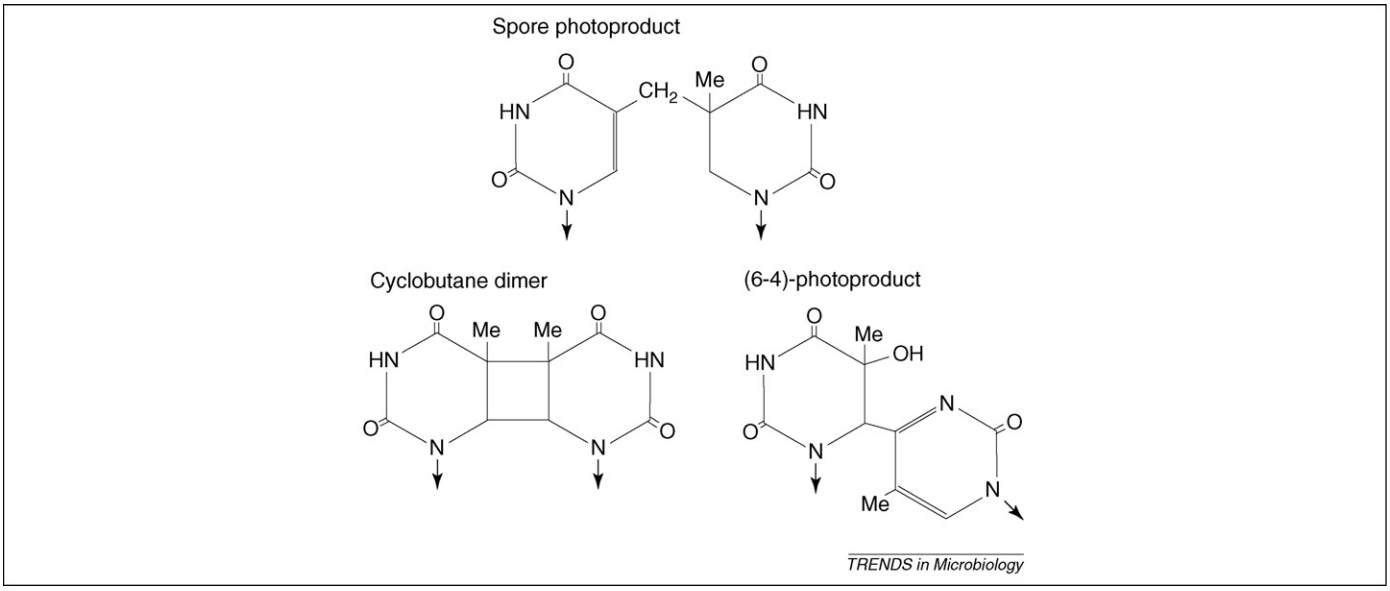

Figure 3. Structures of UV photoproducts formed in DNA in spores. The photoproducts shown are those formed between adjacent thymine residues on the same DNA strand. 'Me' denotes a methyl group and the arrowheads denote bonds to sugar residues of nucleotides. Figure adapted, with permission, from Ref. [5].

resistance to dry heat [12]. Much of the killing of $\alpha^-\beta^-$ spores by wet heat is caused by DNA depurination, and rates of DNA depurination in $\alpha^-\beta^-$ spores are close to rates of DNA depurination *in vitro* [5,26]. UV irradiation of DNA in spores does not give the cyclobutane-type pyrimidine dimers (Py<>Py) and (6–4)-photoproducts (64PP) formed between adjacent pyrimidine residues in DNA in growing cells, but rather an intrastrand thyminyl-thymine adduct termed the spore photoproduct (SP; Figure 3 and Table 2) [5–7,27]. Although SP is a potentially lethal photoproduct, it is repaired early in spore outgrowth in part by an error-free SP-specific repair mechanism that is RecA-independent, and also by RecA-dependent processes. Compared with UV-irradiated wild-type spores, the DNA from UV-irradiated $\alpha^-\beta^-$ spores contains less SP and a large amount of Py<>Py and 64PP (Table 2), and production of at least Py<>Py is a major reason for the UV sensitivity of $\alpha^-\beta^-$ spores [6,27]. Ca-DPA also has effects on spore DNA photochemistry (Table 2). UV irradiation of *Escherichia coli* engineered to have high levels of an α/β-type SASP also gives less Py<>Py and more SP than in *E. coli* that lack this protein [28]. Synthesis of high levels of α/β-type SASP in *E. coli* also causes nucleoid condensation, mutagenesis and cell killing [28,29].

Table 2. UV photoproduct yields in spores of various strains and in DNA with or without α/β-type SASP or Ca-DPA[a,b]

Samples irradiated	Photoproduct		
	Py<>Py	64PP	SP
Wild-type spores	≤1	≤0.1	252
$\alpha^-\beta^-$ spores	103	79	108
DPA-less spores	9	1.2	23
$\alpha^-\beta^-$ DPA-less spores	108	95	4
Naked DNA	176	56	18
DNA plus Ca-DPA (1.5 mm)	39	4.5	15
DNA plus α/β-type SASP	14	≤0.6	29

[a]Data are for wild-type *Bacillus subtilis* spores suspended in water and for dry DNA with or without various additions (see Refs [18,27]). Values are for all Py<>Py and 64PP formed between adjacent pyrimidines on the same DNA strand. SP is formed only between adjacent thymidine residues.
[b]Photoproducts are measured in lesions per 10^4 bases per J cm^{-2}.

Effects of α/β-type SASP on DNA *in vitro* and vice versa

Most of the effects of α/β-type SASP on DNA in spores are also seen *in vitro* using purified DNA and proteins, including α/β-type SASP from *Bacillus* and *Clostridium* species and proteins that are normally abundant and less abundant [4,6,7]. All wild-type α/β-type SASP have similar effects on DNA *in vitro*, including slowing DNA depurination due to wet or dry heat, protecting DNA against cleavage by enzymes, hydroxyl radicals and hydrogen peroxide, preventing cytosine deamination to uracil and markedly changing the UV photochemistry of DNA [4–7,14,15,18,27,30] (Table 2).

In addition to protecting DNA, α/β-type SASP binding stiffens DNA and eliminates sequence-dependent bends, thus increasing the persistence length of DNA [31]. Binding of these proteins also introduces supertwists into relaxed or nicked circular DNA [31,32]. Similar effects of α/β-type SASP on DNA topology are seen on plasmid DNAs from spores [33].

The binding of DNA to α/β-type SASP also has dramatic effects on the properties of the protein because α/β-type SASP change from a random coil in their free state to ~60% α-helix upon DNA binding [34,35]. This change in α/β-type SASP structure slows oxidation of a methionine (emboldened in yellow; Figure 2) and deamidation of an asparagine (emboldened in purple; Figure 2); either of these alterations also reduces DNA binding by the modified protein [36,37]. DNA binding also protects α/β-type SASP against these oxidation and deamidation events in spores [36,37]. Binding to DNA also greatly decreases α/β-type SASP susceptibility to proteases *in vitro* and *in vivo*, including, most importantly, the protease that initiates α/β-type SASP degradation in spore outgrowth (see later) [38–40].

Features of α/β-type SASP-DNA binding and the structure of the complex

All α/β-type SASP bind better to GC-rich DNAs, in particular to polydG–polydC, with polydA–polydT bound

poorly if at all [14,15,34,35,39]. dG_6–dC_6 is bound but binding is generally stronger to larger DNAs, especially for oligodATs. Mixed sequence DNAs such as plasmids can be saturated with α/β-type SASP, with apparent affinity constants as high as $10^7\ M^{-1}$, although this value varies between different proteins [34,35,39]. The site size for α/β-type SASP on DNA is 4–5 bp, binding is similar between pH 6–8, does not require divalent cations and can be insensitive to salt up to 0.4 M [14,15,33–35,39]. Protein binding to DNA is also cooperative and the N-terminal regions of bound proteins at the very least interact with adjacent bound proteins [34,35,41].

As noted earlier, there are major changes in the properties of DNA and protein in α/β-type SASP-DNA complexes and, presumably, substantial changes in the structures of both components (e.g. the α-helical nature of the DNA-bound protein). Although a high-resolution structure of an α/β-type SASP-DNA complex is not available, pertinent low-resolution structural data have been obtained. In particular, spectroscopic analyses suggest that DNA in α/β-type SASP–DNA complexes adopts an A-like conformation [14,15]. However, electron microscopy shows that the rise per bp in DNA does not change upon α/β-type SASP binding, suggesting that protein-bound DNA is in a B-like helix [31,42]. Electron microscopy also suggests that the structure of the protein in the complex promotes side-to-side association of α/β-type SASP-saturated DNA filaments into a tightly packed assembly that might be important in some spore DNA resistance properties [42,43].

Interaction of other components with α/β-type SASP-bound DNA in spores

Although the α/β-type SASP alone have striking effects on DNA properties *in vitro*, the situation in spores is likely to be more complex because additional components interact with DNA. These include the essential HBsu protein found at high levels on DNA in growing cells and spores [44], and Ca-DPA. HBsu decreases the persistence length of DNA saturated with α/β-type SASP *in vitro* without altering effects of α/β-type SASP on DNA photochemistry [44]. Perhaps the effect of HBsu on DNA persistence length facilitates the formation of the compact toroidal chromosomal structure of the spore.

Ca-DPA also photosensitizes spore DNA to UV, perhaps because of transfer of energy from an excited triplet state on DPA to DNA [18] (Table 2). This suggests that Ca-DPA closely approaches the DNA bases. Ca-DPA does not bind appreciably to α/β-type SASP *in vitro*, and because levels of Ca-DPA in spores are high (≥0.5 M if all were soluble), affinities between Ca-DPA and DNA saturated with α/β-type SASP do not need to be high to promote interaction between all three components.

Degradation of α/β-type SASP in spore outgrowth

Although α/β-type SASP are essential to spore DNA resistance, these proteins must be removed to enable DNA transcription in spore outgrowth. Two factors are important in protein removal: (i) dissociation of α/β-type SASP from DNA in fully germinated spores; and (ii) degradation of the proteins initiated by an endoprotease, Gpr, which is specific for α/β-type (and γ-type) SASP [45]. Peptidases then degrade the Gpr cleavage products to amino acids that support much protein synthesis and energy metabolism early in spore outgrowth [14–16]. Dissociation of α/β-type SASP from DNA in germinated spores is promoted by the moderate affinity of these proteins for DNA, especially for AT-rich regions and the increase in core water content that accompanies full spore germination (Box 4). In fully germinated spores that lack Gpr, ~50% of the DNA is SASP-free, even though there is no SASP degradation [45]. Strikingly, even with spores that contain Gpr, α/β-type SASP variants with high affinities for DNA are deleterious – these proteins are poorly degraded in outgrowth because they dissociate minimally from DNA and are thus resistant to Gpr digestion [38–40].

Gpr is a tetrameric endoprotease with 46 kDa subunits and is made as an inactive zymogen (termed P_{46}) in the forespore at about the time of SASP synthesis [14–16]. 1–2 h after its synthesis this zymogen is processed to a form termed P_{41} that is active *in vitro* but not in the developing spore. This processing is an intramolecular cleavage that removes 8–15 N-terminal residues, depending on the species, with the sequence around the $P_{46} \rightarrow P_{41}$ autoprocessing site similar to that cleaved in SASP by P_{41} (Figure 1; Figure 2). Both *in vitro* and *in vivo*, $P_{46} \rightarrow P_{41}$ autoprocessing is triggered by reduced hydration, accumulation of Ca-DPA and acidification, all of which take place in forespores at the time of $P_{46} \rightarrow P_{41}$ conversion [46,47]. The reduced core water content when P_{41} is generated then probably prevents SASP degradation in forespores and dormant spores.

The Gpr sequence is conserved in aerobic and anaerobic spore formers, but has neither sequence homology to known proteases nor sequence motifs characteristic of serine, cysteine, metallo- or aspartate proteases; nucleophilic roles for serine, cysteine or metal ions in catalysis have also been ruled out [48]. However, Gpr might be an aspartate protease because mutagenesis of either of two conserved aspartate residues abolishes enzyme activity without disrupting protein structure [49]. The structure in the region with the two putative catalytic aspartate residues in *B. megaterium* P_{46} is also similar to that in the active site of the aspartate protease pepsin [48,49,50].

Concluding remarks and future questions

The α/β-type SASP are the major factor protecting spore DNA from many damaging agents. These proteins are unique and, given their role in protecting DNA in a dormant organism where an entire chromosome is transcriptionally silent, this uniqueness might not be surprising. Indeed, the presence of these proteins in growing cells is extremely deleterious. Although much has been learned about these proteins and their interaction with DNA, several questions remain. These include the following: (i) Why are there so many *ssp* genes in one organism? Although this might provide redundancy for proteins essential for spore survival, why might seven *ssp* genes be needed? (ii) What is the function of the less abundant α/β-type SASP? Different proteins could have different affinities for different DNA sequences, thus promoting complete chromosome coverage.

However, this suggestion has not been experimentally substantiated. (iii) Might *ssp* genes be carried on a transposable element? This would facilitate movement and multiplication of these genes within a genome, but again there are no data to support this idea. (iv) How is the Gpr zymogen P_{46} maintained in an inactive state and then converted to P_{41}? The P_{41} structure differs from that of P_{46}, being less stable and more open [48]. However, a high-resolution structure of P_{41} is not available, and comparison of this structure with that of P_{46} could be informative. (v) How does α/β-type SASP binding to DNA alter the properties of both DNA and protein? This seems to be a key question and will probably require determination of a high-resolution structure of this protein–DNA complex. Thankfully, as a consequence of questions that remain unanswered, there is still work to be done on this fascinating system.

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References

1 Kennedy, M.J. *et al.* (1994) Preservation records of micro-organisms: evidence of the tenacity of life. *Microbiology* 140, 2513–2529
2 Cano, R.J. and Borucki, M. (1995) Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. *Science* 268, 1060–1064
3 Vreeland, R.H. *et al.* (2000) Isolation of a 250 million-year old halotolerant bacterium from a primary salt crystal. *Nature* 407, 897–900
4 Nicholson, W.L. *et al.* (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev.* 64, 548–572
5 Setlow, P. (2006) Spores of *Bacillus subtilis*: their resistance to radiation, heat and chemicals. *J. Appl. Microbiol.* 101, 514–525
6 Setlow, P. (2001) Resistance of spores of *Bacillus* species to ultraviolet light. *Environ. Mol. Mutagen.* 38, 97–104
7 Nicholson, W.L. *et al.* (2005) The solar UV environment and bacterial spore UV resistance: considerations for panspermia and planetary protection. *Mutat. Res.* 571, 249–264
8 Setlow, B. and Setlow, P. (1996) Role of DNA repair in *Bacillus subtilis* spore resistance. *J. Bacteriol.* 178, 3486–3495
9 Loshon, C.A. *et al.* (1999) Formaldehyde kills spores of *Bacillus subtilis* by DNA damage, and small, acid-soluble spore proteins of the α/β-type protect spores against this DNA damage. *J. Appl. Microbiol.* 87, 8–14
10 Tennen, R. *et al.* (2000) Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *J. Appl. Microbiol.* 89, 330–338
11 Salas-Pacheco, J.M. *et al.* (2005) Role of the Nfo (YqfS) and ExoA apurinic/apyrimidinic endonucleases in protecting *Bacillus subtilis* spores from DNA damage. *J. Bacteriol.* 187, 7374–7381
12 Wang, S.T. *et al.* (2006) The forespore line of gene expression in *Bacillus subtilis*. *J. Mol. Biol.* 358, 16–37
13 Gerhardt, P. and Marquis, R.L. (1989) Spore thermoresistance mechanisms. In *Regulation of Prokaryotic Development* (Smith, I., Slepecky, R.A. and Setlow, P., eds), pp. 43–63, American Society for Microbiology
14 Setlow, P. (1994) Mechanisms which contribute to the long-term survival of spores of *Bacillus* species.. *J. Appl. Bacteriol.* 76 (Symposium Supplement), 49S–60S
15 Setlow, P. (1995) Mechanisms for the prevention of damage to the DNA in spores of *Bacillus* species. *Annu. Rev. Microbiol.* 49, 29–54
16 Driks, A. (2002) Proteins of the spore core and coat. In Bacillus subtilis and its Closest Relatives: from Genes to Cells Sonenshen, A.L., Hoch, J.A. and Losick, R., eds), pp. 527–536, ASM Press
17 Setlow, B. *et al.* (2006) Role of dipicolinic acid in the resistance and stability of spores of *Bacillus subtilis*. *J. Bacteriol.* 188, 3740–3747
18 Douki, T. *et al.* (2005) Photosensitization of DNA by dipicolinic acid, a major component of spores of *Bacillus* species. *Photochem. Photobiol. Sci.* 4, 591–597
19 Raju, D. *et al.* (2006) Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. *BMC Microbiol.* 6, 50
20 Raju, D. *et al.* Antisense RNA-mediated decreased synthesis of small, acid-soluble spore proteins leads to decreased resistance of *Clostridium perfringens* spores to moist heat and UV radiation. *Appl. Environ. Microbiol.* (in press)
21 Carrillo-Martinez, Y. and Setlow, P. (1994) Properties of *Bacillus subtilis* small, acid-soluble, spore proteins with changes in the sequence recognized by their specific protease. *J. Bacteriol.* 176, 5357–5363
22 Setlow, B. *et al.* (2000) Effects of major spore-specific DNA binding proteins on *Bacillus subtilis* sporulation and spore properties. *J. Bacteriol.* 182, 6906–6912
23 Bagyan, I. *et al.* (1998) New small, acid soluble proteins unique to spores of *Bacillus subtilis*: identification of the coding genes and studies of the regulation and function of two of these genes. *J. Bacteriol.* 180, 6704–6712
24 Setlow, B. *et al.* (1998) Small, acid-soluble spore proteins of the α/β-type do not protect the DNA in *Bacillus subtilis* spores against base alkylation. *Appl. Environ. Microbiol.* 64, 1958–1962
25 del Carmen Huesca Espitia, L. *et al.* (2002) Base-change mutations induced by various treatments of *Bacillus subtilis* spores with and without DNA protective small, acid-soluble spore proteins. *Mutat. Res.* 503, 77–84
26 Setlow, B. and Setlow, P. (1994) Heat inactivation of *Bacillus subtilis* spores lacking small, acid-soluble spore proteins is accompanied by generation of abasic sites in spore DNA. *J. Bacteriol.* 176, 2111–2113
27 Douki, T. *et al.* (2005) Effects of the binding of α/β-type small, acid-soluble spore proteins on the photochemistry of DNA in spores of *Bacillus subtilis* and *in vitro*. *Photochem. Photobiol.* 81, 163–169
28 Setlow, B. *et al.* (1991) Synthesis of a *Bacillus subtilis* small, acid-soluble spore protein in *Escherichia coli* causes cell DNA to assume some characteristics of spore DNA. *J. Bacteriol.* 173, 1642–1653
29 Setlow, J.K. *et al.* (1992) Mutation and killing of *Escherichia coli* expressing a cloned *Bacillus subtilis* gene whose product alters DNA conformation. *J. Bacteriol.* 174, 2943–2950
30 Sohail, A. *et al.* (2002) Protection of DNA by α/β-type small, acid-soluble proteins from *Bacillus subtilis* spores against cytosine deamination. *Biochemistry* 41, 11325–11330
31 Griffith, J. *et al.* (1994) Electron microscopic studies of the interaction between a *Bacillus* α/β-type small, acid-soluble spore protein with DNA: protein binding is cooperative, stiffens the DNA and induces negative supercoiling. *Proc. Natl. Acad. Sci. U. S. A.* 91, 8224–8228
32 Nicholson, W.L. *et al.* (1990) Binding of DNA *in vitro* by a small, acid-soluble spore protein and its effect on DNA topology. *J. Bacteriol.* 172, 6900–6906
33 Nicholson, W.L. and Setlow, P. (1990) Dramatic increase in the negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis*. *J. Bacteriol.* 172, 7–14
34 Hayes, C.S. *et al.* (2000) Equilibrium and kinetic binding interactions between DNA and a group of novel, non-specific DNA binding proteins from spores of *Bacillus* and *Clostridium* species. *J. Biol. Chem.* 275, 35040–35050
35 Hayes, C.S. *et al.* (2001) N-terminal amino acid residues mediate protein-protein interactions between DNA-bound α/β-type small, acid-soluble spore proteins from *Bacillus* spores. *J. Biol. Chem.* 276, 2267–2275
36 Hayes, C.S. *et al.* (1998) *In vitro* and *in vivo* oxidation of methionine residues in small, acid-soluble spore proteins from *Bacillus* species. *J. Bacteriol.* 180, 2694–2700
37 Hayes, C.S. and Setlow, P. (1997) Analysis of deamidation of small, acid-soluble spore proteins from *Bacillus subtilis in vitro* and *in vivo*. *J. Bacteriol.* 179, 6020–6027
38 Setlow, B. and Setlow, P. (1995) Binding to DNA protects α/β-type small, acid-soluble spore proteins of *Bacillus* and *Clostridium* species

against digestion by their specific protease as well as other proteases. *J. Bacteriol.* 177, 4149–4151
39 Kosman, J. and Setlow, P. (2003) Effects of carboxyl-terminal modifications and pH on the binding of a *Bacillus subtilis* small, acid-soluble spore protein to DNA. *J. Bacteriol.* 185, 6095–6103
40 Hayes, C.S. and Setlow, P. (2001) An α/β-type small, acid-soluble spore protein which has a very high affinity for DNA prevents outgrowth of *Bacillus subtilis* spores. *J. Bacteriol.* 183, 2662–2666
41 Hayes, C.S. and Setlow, P. (1998) Identification of protein-protein contacts between α/β-type small, acid-soluble spore proteins of *Bacillus* species bound to DNA. *J. Biol. Chem.* 273, 17326–17332
42 Frenkiel-Krispin, D. *et al.* (2004) Structure of DNA–SspC complex: implications for DNA packaging, protection and repair in bacterial spores. *J. Bacteriol.* 186, 3525–3530
43 Frenkiel-Krispin, D. and Minsky, A. (2006) Nucleoid organization and maintenance of DNA integrity in *E. coli*, *B. subtilis* and *D. radiodurans*. *J. Struct. Biol.* 156, 311–319
44 Ross, M.A. and Setlow, P. (2000) The *Bacillus subtilis* HBsu protein modifies the effects of α/β-type small, acid-soluble spore proteins on DNA. *J. Bacteriol.* 182, 1942–1948
45 Sanchez-Salas, J.L. *et al.* (1992) Properties of mutants of *Bacillus megaterium* and *Bacillus subtilis* which lack the protease that degrades small, acid-soluble proteins during spore germination. *J. Bacteriol.* 174, 807–814
46 Illades-Aguiar, B. and Setlow, P. (1994) Autoprocessing of the protease that degrades small, acid-soluble proteins of spores of *Bacillus* species is triggered by low pH, dehydration and dipicolinic acid. *J. Bacteriol.* 176, 7032–7037
47 Sanchez-Salas, J.L. and Setlow, P. (1993) Proteolytic processing of the protease which initiates degradation of small, acid-soluble proteins during germination of *Bacillus subtilis* spores. *J. Bacteriol.* 175, 2568–2577
48 Nessi, C. *et al.* (1998) Structure and mechanism of action of the protease that degrades small, acid-soluble spore proteins during germination of spores of *Bacillus* species. *J. Bacteriol.* 180, 5077–5084
49 Carroll, T.M. and Setlow, P. (2005) Site-directed mutagenesis and structural studies suggest that the germination protease, GPR, in spores of *Bacillus* species is an atypical aspartic acid protease. *J. Bacteriol.* 187, 7119–7125
50 Ponnuraj, K. *et al.* (2000) Crystal structure of a novel germination protease from spores of *Bacillus megaterium*: structural arrangement and zymogen activation. *J. Mol. Biol.* 300, 1–10
51 Setlow, P. and Johnson, E.A. (2007) Spores and their significance. In *Food Microbiology, Fundamentals and Frontiers* (Doyle, M.P. and Beuchat, L.R., eds), pp. 35–67, ASM Press
52 Kim, H. *et al.* (2006) The *Bacillus subtilis* spore coat interaction network. *Mol. Microbiol.* 59, 487–502
53 Klobutcher, L.A. *et al.* (2006) The *Bacillus subtilis* spore coat provides "eat resistance" during phagosomal predation by the protozoan *Tetrahymena thermophila*. *Proc. Natl. Acad. Sci. U. S. A.* 103, 165–170
54 Westphal, A.J. *et al.* (2003) Kinetics of size changes of individual *Bacillus thuringiensis* spores in response to changes in relative humidity. *Proc. Natl. Acad. Sci. U. S. A.* 100, 3461–3466
55 Cortezzo, D.E. and Setlow, P. (2005) Analysis of factors influencing the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. *J. Appl. Microbiol.* 98, 606–617
56 Cowan, A.E. *et al.* (2004) Lipids in the inner membrane of dormant spores of *Bacillus* species are immobile. *Proc. Natl. Acad. Sci. U. S. A.* 101, 7733–7738
57 Cowan, A.E. *et al.* (2003) A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4209–4214
58 Setlow, P. (2003) Spore germination. *Curr. Opin. Microbiol.* 6, 550–556