MINIREVIEW

I Will Survive: Protecting and Repairing Spore DNA

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INTRODUCTION

The process of sporulation in *Bacillus* species is generally initiated upon the depletion of one or more nutrients, i.e., when the environment is no longer conducive to survival of an actively metabolizing cell. The end product of sporulation is an endospore with no detectable metabolism, which lacks most common high-energy compounds, including ATP and other nucleoside triphosphates (28). However, this dormant spore can survive for long periods of time even in a hydrated state, as there are reports of spores which may have survived several thousand years (33). While there are no direct experimental measurements of the percentage of spores which survive over such extended periods, observation of >50% survival of spores stored in water at 6 to 10°C for at least a year is not uncommon. Clearly, in order for spores to survive during these long periods of dormancy, mechanisms must be operating to prevent accumulation of potentially lethal damage in spore DNA. Although damage to essential proteins (e.g., RNA polymerase) could result in spore killing, if a spore has a thousand molecules of such a protein almost all would have to be inactivated to cause spore death. In contrast, a spore with its single chromosome can acquire a lethal mutation through one change in an essential gene. (This argument is a bit simplistic in that spores of some species can have multiple [two to four] genomes.)

In order to meet the challenge of protecting its DNA from irreversible damage during long-term dormancy, a spore faces at least two problems more severe than those encountered by growing cells. The first problem is the time for which DNA in the spore must be protected. In a cell growing at 37°C, the potential for accumulation of one lethal lesion in the cell's DNA per 24 h (for example, because of depurination) will be of only minor concern if the cell is doubling every 2 h. In contrast, a spore must survive over much longer time periods, yet it generates no progeny during this time. The second problem is the inability of the dormant spore to constantly repair various types of DNA damage, in contrast to the ongoing repair of such damage in growing cells. While spores may contain enzymes for DNA repair, their action in the dormant spore appears highly unlikely, in large part because of the absence of ATP and deoxynucleoside triphosphates (28). The low water content in the spore core (see below) also undoubtedly limits enzyme activity in this cellular compartment. Thus, DNA damage generated in spores during their dormancy will continually accumulate and will only be repaired when the spore "returns to life" in the process of spore germination as enzyme action and energy metabolism are reinitiated. However, at this time the amount of DNA damage accumulated could overwhelm DNA repair capacities.

There are a number of types of processes which can lead

to DNA damage. These include chemical modification such as alkylation or oxidation, base removal such as by depurination, and damage caused by UV light. Spores appear to have mechanisms which greatly slow the rate of chemical damage to DNA (24), but these mechanisms are not well understood and may involve a very low spore permeability to DNA-damaging agents (9). Consequently, this review will concentrate on mechanisms that spores utilize to prevent DNA damage due to base removal (i.e., depurination) and UV radiation. Most discussion will focus on work with *Bacillus subtilis* because of the utility of molecular genetic approaches in understanding this organism's DNA protection mechanisms. However, it seems likely that these mechanisms will be similar in spores of related species; therefore, relevant work with other organisms will be noted. While protection of the DNA in *B. subtilis* spores from irreversible damage (defined as changes resulting in the inability to generate progeny) due to depurination and UV radiation is not yet understood completely, it will be the thesis of this review that spores have evolved three distinct strategies for dealing with these potential types of DNA damage. (i) The greatly decreased water content in the spore core retards and/or alters chemical reactions affecting DNA. (ii) The binding of spore DNA by a unique group of proteins, termed α/β -type small, acid-soluble proteins (SASP), further slows and/or alters the DNA's reactivity. (iii) DNA repair processes act in the early minutes of spore germination to repair DNA damage accumulated during dormancy.

DNA DAMAGE

Depurination. Measurements of the rate of DNA depurination in vitro (14) allow the calculation that the *B. subtilis* spore genome should lose ~10 purines per day at 10°C (with the rate of pyrimidine loss more than 25-fold slower [13]). (This value was calculated by using 5×10^6 bp as the size of the genome [11], an internal spore pH of ~6.3 [26], and data for rates of DNA depurination in vitro [14].) This depurination rate would lead to the loss of 0.07% of total spore DNA purines (3,500 purines per chromosome) per year at this temperature, yet *B. subtilis* spore populations exhibit >50% survival over such a period (32). Spores of *B. subtilis* also exhibit >50% survival for 1 day at 60°C (6, 23), a condition which again should lead to 0.07% depurination. While the amount of depurination required to generate a lethal event in spores is not clear, a gene on a transforming DNA fragment which has undergone <0.05% depurination in vitro is inactivated (14). Since spores exhibit no killing under conditions which in vitro should give even greater depurination, there must be mechanisms to slow the rate of such DNA damage in vivo. However, there is evidence that DNA depurination can be significant in spores under some conditions. Heating

TABLE 1. Survival of *B. subtilis* spores after UV or heat treatment^a

Spore or cell type	UV resistance ^b	UV photo-product		Heat treatment ^c	
		T()T	SP	Survival (%)	Survivors with mutations (%) ^d
Wild-type spores	315	No	Yes	79	<0.2
$\alpha^- \beta^-$ spores	25	Yes ^e	Yes ^f	1	12
Wild-type or $\alpha^- \beta^-$ vegetative cells	40	Yes	No	<10 ⁻⁴	NT

^a Data are taken from references 6, 20, and 30.^b The value given is the UV dose (joules per square meter) at 254 nm required to give 90% killing of a population.^c The treatment was 85°C for 30 min.^d Survivors were screened for asporogenous and auxotrophic mutations. NT, not tested.^e The level of T()T was one-half that obtained in vegetative cells with the same UV dose.^f The level of SP was one-half that obtained in wild-type spores with the same UV dose.

B. subtilis spores, either dry or hydrated, at temperatures giving significant killing results in a high percentage (5 to 20%) of readily observed mutations in the survivors (12, 21, 39). The high percentage of mutations in the survivors of these heat treatments suggests that the spores that were killed also carried mutations, possibly more than in the survivors but certainly at least one lethal mutation. These heat-induced mutations have been suggested to be the result of DNA depurination (12, 21, 39), although this has not been proven. However, this work does indicate that DNA can be the target for heat killing of spores, possibly by depurination, although disruption of a DNA-protein complex which then leads to depurination could also be involved. Note that while DNA depurination is a problem that *B. subtilis* spores must deal with, this should not be a major problem in growing cells of this organism (although it may be in cells of thermophiles), first because they grow and second because temperatures giving a high rate of depurination in vivo probably kill cells for reasons not having to do with the DNA (e.g., breakdown of membrane permeability).

UV irradiation. Spores of *B. subtilis* are 10 to 50 times more resistant to UV in the 254-nm range than are growing cells (Table 1) (30). Similar elevated UV resistance is observed in spores of other *Bacillus* species (30). The elevated UV resistance of *B. subtilis* spores is not surprising, because *B. subtilis*, a soil organism, runs the risk of constant exposure to UV light. UV irradiation of DNA in vitro or in growing cells generates a number of DNA lesions, including cyclobutane-type dimers between adjacent pyrimidines; the most abundant of these are *cis,syn*-thymine dimers (T()T) (Fig. 1), followed by dimers between cytosine and thymine residues (C()T) and between adjacent cytosines (C()C) (7).

FIG. 1. Structures of SP and a T()T dimer. The structures shown lack the base-sugar bond.

Bipyrimidine photoadducts, including the 6-4 photoproducts, are also formed (37). While cells have a variety of mechanisms to repair these types of UV damage, any one of these lesions can be lethal. Since spores cannot repair UV damage in an ongoing manner, such lesions would be expected to accumulate in spore DNA, possibly overwhelming the capacity for DNA repair early in spore germination. The reason for the elevated spore UV resistance is a difference in spore DNA's UV photochemistry in vivo (30), such that UV irradiation of dormant spores generates no T()T (and probably no C()T or C()C), but rather causes formation of a thymine-thymine adduct termed spore photoproduct (SP) (Fig. 1). Some formation of 6-4 photoproducts by UV irradiation of spores has been reported (15); however, there is no information on the yield of this type of photoproduct in spores as a function of UV fluence, and it may be much lower than in growing cells. In contrast, the formation of SP in spores as a function of UV fluence is only slightly less than that of T()T formation in growing cells (30). Proof that the altered DNA photochemistry is the cause of spore UV resistance came with the demonstration that spores in which T()T dimers are formed upon UV irradiation (see below) are even more UV sensitive than growing cells (Table 1) (30). The reason that formation of SP in a spore's DNA is less lethal than T()T formation in a cell's DNA is that spores have at least two repair systems for SP, one of which is unique to SP (18, 38).

SPORE DNA PROTECTION MECHANISMS

As discussed above, there are several types of DNA damage which spores must avoid in order for their extended survival. As may not be surprising, there appear to be a number of mechanisms used by spores to protect their DNA against such damage.

Decreased water content. Although spores can survive for extended periods in water, there is much evidence that the spore core or protoplast (the site of spore DNA!) has a decreased water content (28 to 57% of wet weight) relative to that in a growing cell ($\geq 80\%$) (2, 10). Although there is a lack of detailed knowledge of both how this reduction in spore core water content is achieved during sporulation and the percentage of total core water which is free water (likely to be the most important parameter), the amount of core water is clearly an important variable in determining spore survival at various temperatures. Thus, analysis of spores from many *Bacillus* species indicates that those with less core water almost invariably exhibit greater survival at elevated temperatures (i.e., heat resistance) than do those with more core water. It seems likely that spores with less core water will also survive longer than spores with more at rather low temperatures (10 to 25°C). While this latter postulate has never been tested experimentally, plots of the log of the rate of spore killing versus temperature (generally at temperatures of $>60^\circ\text{C}$) are relatively linear (23). Accumulation of divalent cations by spores also increases spore survival at elevated temperatures, but it is not clear if mineralization simply reduces the amount of free water in the spore core or has other effects on spore macromolecules as well (10).

It appears likely that reduced spore core water can reduce or alter DNA damage in a number of ways. First, it should slow the rate of DNA depurination, which is largely water catalyzed at neutral pH (14), and it may also slow subsequent DNA strand cleavage at the resulting abasic site. Indeed, many chemical reactions involving DNA might be slowed by the decrease in spore core water. Second, a

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sspA: ANNNSGNSNNLLVPGAQAID**QMKLEI**ASEFGVNLGADTTSRANGSVGG**EITKRLVSFAQQNMGGGQF**

sspB: ANQNSSNDLLVPGAQAID**QMKLEI**ASEFGVNLGADTTSRANGSVGG**EITKRLVSFAQQQMGGRVQ**

sspC: AQQSRSRNNNDLLIPQAASA**IEQMKLEI**ASEFGVNLGAETTSRANGSVGG**EITKRLVRLAQQNMGGQFH**

sspD: ASRNKLVVPGVEQALD**QFKLEV**ASEFGVNLGSDTVARANGSVGG**EMTKRLVQQAQSQNLNGTTK**

FIG. 2. Amino acid sequences of *B. subtilis* α/β -type SASP. Primary sequences of the four *B. subtilis* α/β -type SASP known are given in the one-letter code, without the N-terminal methionine which is removed posttranslationally. The *sspA* and *sspB* genes code for the two major α/β -type SASP (α and β), while the *sspC* and *sspD* genes code for minor species. The amino acids conserved in all known α/β -type SASP from the *Bacillus* line of sporeformers are in boldface type; residues which are similar in all these proteins are underlined. The vertical arrow denotes the site of cleavage by a spore-specific endoprotease. The data are taken from references 3 and 29.

decrease in DNA hydration would be expected to cause some change in the DNA's UV photochemistry, as UV irradiation of poorly hydrated DNA in vitro produces two- to threefold less T(→T than does UV irradiation of well-hydrated DNA and also generates significant SP (20, 22) (note, however, that T(→T is still formed in dry DNA!). Third, the reduced amount of free core water may promote interaction of spore DNA with a group of proteins termed α/β -type SASP (see below), which exert a number of protective effects on DNA (27, 29, 30).

α/β -type SASP. The DNA in dormant spores of *B. subtilis* is complexed with a group of unique proteins, the α/β -type SASP, which are synthesized only within the developing spore at approximately the third hour of sporulation (8, 29). There are two major α/β -type SASP in *B. subtilis* (termed α and β), as well as a number of minor ones (29). While *B. subtilis* α/β -type SASP are products of unique genes (termed *ssp*), their amino acid sequences are almost identical, with 35 identical and 11 similar residues in these small (68- to 71-residue) proteins (Fig. 2) (29). The amino acid sequences of α/β -type SASP have also been extremely highly conserved in other *Bacillus* species (Fig. 2), as well as in *Clostridium* species (3). However, they exhibit no significant sequence similarity to any other protein in available data bases. The precise functions of the conserved residues in α/β -type SASP have not yet been definitively established. However, it appears likely that the highly conserved region in the carboxyl-terminal half of these proteins is involved in DNA binding (35). At least one function of the conserved residues in the amino-terminal half of the proteins is to allow their recognition and cleavage by a specific endoprotease (Fig. 2, arrow; see below) (29).

α/β -type SASP are bound to spore DNA in vivo and are double-stranded DNA-binding proteins in vitro (8, 19, 27). They exhibit no particular specificity for different double-stranded DNAs, save that the DNA must be capable of adopting an A-like conformation (27). Indeed, these proteins cause a change in DNA to an A-like conformation in vitro and almost certainly do so in vivo as well (16, 31). α/β -type SASP are synthesized in sporulation in parallel with the acquisition of UV resistance by the developing spore and the change in the spore DNA's UV photochemistry from T(→T to SP formation (1, 30). These proteins are rapidly degraded to amino acids early in spore germination, and their degradation is initiated by a single cleavage by a sequence-specific endoprotease termed GPR, which is also specific to the spore stage of the *B. subtilis* life cycle (Fig. 2) (25, 34).

The absence of most α/β -type SASP from dormant spores

through deletion of genes for SASP- α and - β results in UV-sensitive spores in which UV irradiation produces significant T(→T (Table 1); these mutant spores (termed $\alpha^- \beta^-$) also exhibit a significant loss in heat resistance (Table 1), although they exhibit no change in spore core water content (30). Strikingly, a large percentage of the survivors of a heat treatment giving ~99% killing of $\alpha^- \beta^-$ spores carry easily observed mutations (Table 1). This suggests that in vivo α/β -type SASP not only alter the type of UV damage to DNA but also prevent heat damage, possibly by slowing DNA depurination. These defects in $\alpha^- \beta^-$ spores can be cured completely or almost completely by synthesis of sufficient levels of either SASP- α or - β , or even of a minor α/β -type SASP (29, 30, 35); these proteins appear to be largely interchangeable in vivo. However, mutant α/β -type SASP which no longer bind DNA cannot restore the UV or heat resistance of $\alpha^- \beta^-$ spores to wild-type levels (35).

As noted above, α/β -type SASP are double-stranded DNA-binding proteins which cause DNA to change to an A-like conformation. The affinity between these proteins and DNA is not great (K_D , 10^{-6} to 10^{-5}), but their concentration in vivo is high (>1 mM), and there are sufficient amounts in spores to saturate the DNA (19, 27, 29). α/β -type SASP-DNA interaction may also be promoted by the low free water content in the spore core. Studies of the effect of α/β -type SASP binding on DNA properties in vitro have shown that this binding protects the DNA backbone from chemical and enzymatic cleavage (27) and reduces greatly (>20-fold) the rate of depurination (6). This latter observation may explain the role of these proteins in spore heat resistance. Studies of DNA's UV photochemistry in vitro have shown that α/β -type SASP binding abolishes formation of all cyclobutane-type dimers (C(→C, C(→T, and T(→T) and promotes SP formation (5, 20). UV-induced production of 6-4 photoproducts in DNA in vitro is also blocked by α/β -type SASP binding (5). Consequently, the presence of this novel group of proteins on spore DNA alters DNA properties significantly to allow for long-term survival. In one case this appears to be by slowing DNA's chemical reactivity and thus slowing depurination. In a second case, α/β -type SASP binding alters DNA's photochemistry such that SP is the predominant photoproduct.

Repair systems. As noted above, one effect of α/β -type SASP binding is to alter DNA's UV photochemistry, such that SP is produced rather than cyclobutane dimers. While SP is potentially a lethal lesion in DNA, spores have mechanisms to repair this lesion—indeed, they have two such systems (17, 18, 37). One appears to be the excision

repair system, present in growing cells, which can also repair cyclobutane dimers. However, spores have a second repair system which operates only on SP and is specific to the spore stage of the *B. subtilis* life cycle. The exact mechanism of this rather error-free SP-specific repair system is not clear, but it appears to involve monomerization of an SP back to two thymines early in spore germination in a process requiring energy but not light (38). This SP repair system is defined by the *spe* (formerly *ssp*) locus (17). Mutations in both SP repair pathways result in extremely UV-sensitive spores.

The SP-specific repair system is the only spore-specific DNA repair system identified to date. However, given the DNA damage that may accumulate in spores during their potentially long dormancy, spores may well contain high levels of enzymes for repair of other types of DNA damage, including oxidized bases and abasic sites resulting from depurination (4, 36). Indeed, there can be significant DNA repair synthesis early in spore germination (28). Analysis of DNA repair systems in dormant and germinating spores could be a fruitful area for future study.

CONCLUSION

Faced with the need to preserve its DNA's integrity over long periods of time, spores of *B. subtilis* and other *Bacillus* species have evolved a number of protective mechanisms. Protection against DNA depurination involves a decreased water content in the spore core, as well as synthesis of a special class of DNA-binding proteins, the α/β -type SASP. These adaptations may greatly slow spore DNA depurination in vivo. It is also possible that spores rapidly repair this type of DNA damage early in spore germination. Protection against accumulation of lethal UV damage involves synthesis of α/β -type SASP whose association with DNA may be promoted by the spore core's low water content. The presence of the spore chromosome in an α/β -type SASP-DNA complex results in production only of SP upon UV irradiation, and SP is repaired by a process much more error free than is cyclobutane dimer repair. This SP-specific repair system is unique to spores and operates only during spore germination. Although essentially nothing is known about protection of spore DNA from oxidative damage, it appears likely that the reduced spore core water content would also slow the rate of such damage, as would the presence of the α/β -type SASP (27). There could also be spore-specific repair systems for oxidative DNA damage. While bacterial spores may be dormant, they remain subjects for active study.

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