Accelerated Publications

Protection of DNA by α/β-Type Small, Acid-Soluble Proteins from *Bacillus subtilis* Spores Against Cytosine Deamination†

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ABSTRACT: Spores of *Bacillus subtilis* contain high levels of proteins, termed α/β-type small, acid-soluble proteins (SASP), that protect the spore's DNA against different types of DNA damage. We tested one such protein, SspC, and two of its variants for their ability to protect plasmid DNA against hydrolytic deamination of cytosine to uracil. If unrepaired, such damage to DNA causes C to T mutations. We found that one SspC variant, $SspC^{\Delta 11-D13K}$, protected DNA against cytosine deamination at two different temperatures (45 and 70 °C) and pH values (5.2 and 7.9), reducing the rate of deamination by as much as 10-fold. At 70 °C, pH 7.9, the wild-type SspC and its variant, $SspC^{\Delta 11}$, provided little protection against deamination but were effective in protecting DNA at 45 °C, pH 7.9. Parallel studies of the abilities of these proteins to protect DNA against restriction digestion revealed that there was a good correlation between the abilities of the proteins to protect against restriction endonucleases and reductions in cytosine deaminations. These results show that the binding of SspC variants to DNA can prevent attack on DNA bases by water and suggest a new general mechanism by which DNA-binding proteins in cells may be able to protect chromosmes from endogenous and exogenous reactive chemicals by excluding them from the vicinity of DNA.

Cellular DNA is damaged by several endogenous chemicals, including water, reactive oxygen species, and S-adenosylmethionine (*1*, *2*). These insults cause mutations and genomic instabilities that may contribute to a variety of human genetic diseases. In particular, hydrolytic deamination of cytosine to uracil is the principal cause of C to T mutations (*3*), and similar deamination of 5-methylcytosine (5meC) to thymine is probably responsible for the occurrence of mutational hotspots at 5meCs in DNA. Although DNA repair processes are known in both prokaryotes and eukaryotes that repair U·G and T·G mismatches to C·G (*4*−*6*), deaminations of C's and 5meC's are thought to make a major contribution to disease-causing human mutations (*7*, *8*).

Because water has been an integral part of living organisms since the beginning of life on Earth, it is reasonable to assume that cells have developed protective mechanisms to reduce cytosine deamination. A possible mechanism for the protection of DNA against cytosine deamination is to shield it from the aqueous environment. We wondered whether DNA-binding proteins could do this job effectively. Protein−DNA complexes do contain bound water molecules, but many of these are immobile and are unlikely to initiate an attack on cytosines unless they are properly positioned and oriented. The logic of this DNA protection mechanism is so simple that it would be surprising if cells have not evolved to take advantage of it. Additionally, there is precedent for such a role for DNA-binding proteins. *Escherichia coli* photolyase protects cytosines in T−C photodimers against deamination if the enzyme is allowed to bind the dimers, but not repair them (*9*). We decided to test this idea using proteins from bacterial spores that are known to protect DNA from external agents.

The α/β-type small, acid-soluble proteins (SASP) are a group of nonspecific DNA-binding proteins of similar amino acid sequence present at very high levels in spores of *Bacillus*, *Clostridium*, and other closely related gram-positive bacteria (*10*, *11*). The α/β-type SASPs accumulate to very high levels in spores, levels that are sufficient to saturate the spore DNA (*11*). These proteins bind cooperatively to DNA in vitro, and DNA saturated with α/β-type SASPs is straightened and stiffened by them to adopt an A-like helical structure both in vivo and in vitro (*10*, *11*). In *Bacillus* species, two major α/β-type SASP are present at very high levels in the spore, while several minor α/β-type SASP are present at much lower levels (*11*). Binding of these proteins to the spore chromosome plays an important role in long-term spore survival and is a significant factor in the resistance of spore DNA to damage caused by wet and dry heat, UV

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radiation, and genotoxic chemicals (*10*). Indeed, *Bacillus subtilis* spores lacking the major α/β-type SASP are much more sensitive to many types of DNA damaging agents than are wild-type spores (*12*).

Purified α/β-type SASP bind DNA nonspecifically and dramatically reduce the sensitivity of DNA to chemical and physical agents (*11*, *12*). Binding of these proteins to DNA also blocks cleavage of DNA by restriction endonucleases and nonspecific DNAases (*13*). Additionally, a number of α/β-type SASP, including SspC, a minor *B. subtilis* protein, protect DNA against depurination at elevated temperatures both in vitro and in vivo (*13*). However, the effects of α/β-type SASP on cytosine deamination have not been studied. We show here that SspC and its variants do protect DNA against deamination and that this ability of these proteins parallels their ability to bind DNA.

EXPERIMENTAL PROCEDURES

Bacterial Strains. The *E. coli* K-12 strains used in these experiments were GM31 (*dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78 galK2 galE2 xyl-5 thi-1 mtl-1*), BH156 (= GM31 *ung-1 tyrA*::Tn*10*), and BH143 (Δ(*mrr-hsdRMS-mcrBC*) *mcrA* Φ80d*lacZ*Δ*M*15 Δ*lacX74 deoR endA1 araD139* Δ(*ara, leu*)7697 *galU galK rpsL* Δ(*dcm-vsr*) *hisG*::Tn*10*).

Prepararion of Plasmid DNA and SspC Variants. Construction of the plasmid pUP31 containing the *kanS-94D* allele under the transcriptional control of a hybrid UP-*tac* promoter has been described previously (*14*). The plasmid DNA was isolated from GM31 by alkaline lysis in the presence of SDS and was purified further through two rounds of polyethylene glycol precipitation. To study protection of methylated cytosine in DNA, pUP31 DNA was methylated using M.*Eco*RII in the presence of S-adenosylmethionine (SAM) as described previously (*15*). SAM was removed from the mixture using a G-50 Sephadex spin column; the DNA was extracted with phenol−chloroform and precipitated with ethanol.

Construction of strains overexpressing wild-type (WT) SspC or its variants $SspC^{\Delta 11}$ and $SspC^{\Delta 11-D13K}$ and the purification of these proteins have been described (*16*). All proteins were 95% pure, as judged by the staining of proteins run on SDS-PAGE with Coomassie Blue.

Assays for Cytosine Deamination and DNA Protection. To study the rate of increase in C to T mutations, 1 μg of pUP31 DNA was incubated in 20 mM citrate−phosphate buffer (pH 5.2) with or without $SspC^{\Delta 11-D13K}$ (5 μg) at 70 °C for various lengths of time. Assuming that the binding site for α/β-type SASP on DNA is ∼5 base pairs (bp) (*17*), there was 2.5-fold excess of protein over available DNA binding sites. Samples were removed at various times, and reactions were terminated by the addition of SDS to 1.0%, followed by extraction with phenol−chloroform. Following ethanol precipitation, the DNA was electroporated into BH156 (BH143 in the case of methylated DNA), and the cells were plated on LB with 50 μg/mL carbenicillin or LB with 50 μg/mL kanamycin to, respectively, score carbenicillin-resistant ($Carb^R$) and kanamycin-resistant (Kan^R) cells. The revertant frequency was the ratio (total no. of Kan^R cells/total no. of $Carb^R$ cells).

To compare the abilities of SspC variants to protect DNA against restriction endonucleases, 1 μg of DNA was incubated with a 5-fold molar excess over binding sites or without the different proteins in a buffer containing 20 mM Tris-HCl, pH 7.9 (at ambient temperature), 10 mM $MgCl_2$, and 1 mM DTT. Following an incubation for 3 h (at 70 °C) or 18 h (45 °C), the DNA was respectively challenged with *Bsr*DI or *Bst*NI, and the incubations were continued for 30 additional minutes. The resulting DNA fragments were separated by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized with UV light.

In parallel reactions, DNA was incubated for 6 h (70 °C) or 30 h (45 °C) in the absence of restriction enzymes. The buffer used in these reactions was identical to that used for restriction digestion and the reactions either contained one of the SspC variants or no protein. The reactions were terminated with the addition of SDS, the DNA was deproteinized, and it was electroporated into BH156 to determine Kan^R revertant frequency.

RESULTS

Experimental System for the Study of Protection against Deamination of Cytosine. To study the effects of protein binding on cytosine deamination, we used a genetic reversion assay in which an inactive *kan* allele reverts to kan^+ (phenotype- Kan^R) through a C to T mutation in codon 94 (*18*). The *kan* allele resides on a plasmid, and when this plasmid (pUP31) is introduced into cells defective in the removal of uracils in DNA (genotype *ung*), uracils introduced in either the first or the second position of codon 94 by cytosine deamination are replicated causing C to T mutations and a Kan^R phenotype. Consequently, accumulation of uracils in pUP31 increases the frequency of Kan^R revertants.

SspC is an α/β-type SASP that is known to bind DNA nonspecifically (*10*, *16*). Several variants of this protein have been constructed, one of which is missing 11 amino acids near the amino terminus (Gln2 through Asn12) and named $SspC^{\Delta 11}$. The variant $SspC^{\Delta 11-D13K}$ was constructed from $SspC^{\Delta 11}$ by changing Asp13 to Lys (*19*). We used $SspC^{\Delta 11-D13K}$ for our initial studies because it has significantly higher affinity for DNA than wild-type SspC or $SspC^{\Delta 11}$, and the complexes formed between DNA and $SspC^{\Delta 11-D13K}$ are more thermostable (*20*). In our initial experiments, we incubated plasmid pUP31 with and without $SspC^{\Delta 11-D13K}$ under conditions of high temperature and low pH. These extreme reaction conditions were chosen because the rate of cytosine deamination is much higher under these conditions than at physiological pH and 37 °C (*21*), thereby increasing the sensitivity of the DNA protection assay.

Protection of DNA against Cytosine Deamination by $SspC^{\Delta 11-D13K}$. Plasmid pUP31 DNA was incubated at 70 °C, pH 5.2, with excess (in terms of available binding sites) of $SspC^{\Delta 11-D13K}$ for up to 6 h, and the DNA was electroporated into *ung* cells to quantify the frequency of cytosine deamination. In the absence of any protein, the Kan^R revertant frequency increased linearly during the 6 h incubation, becoming ∼60-fold higher than that of the unincubated control (Figure 1). The DNA incubated in the presence of $SspC^{\Delta 11-D13K}$ also suffered deamination, but at a rate that was ∼10-fold lower (Figure 1).

We repeated the incubation of pUP31 with $SspC^{\Delta 11-D13K}$ in the presence of SDS in the reaction to see if denaturation of the protein would eliminate its protection against cytosine

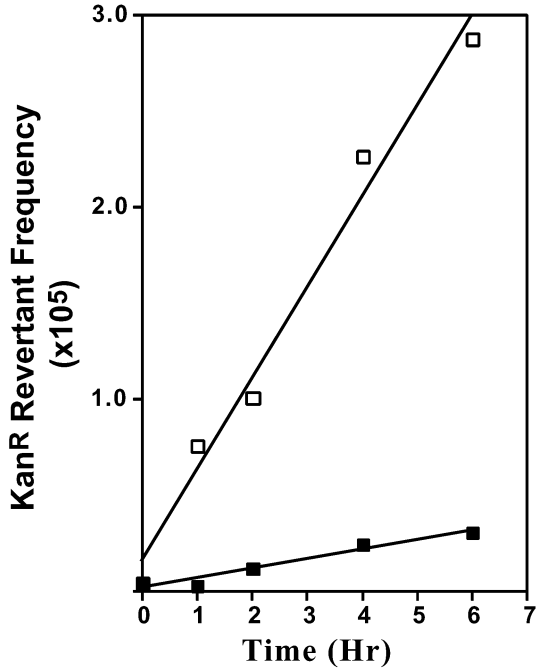

FIGURE 1: Protection of cytosines by SspC$^{\Delta 11D13K}$. Following incubation of pUP31 DNA with or without SspC$^{\Delta 11-D13K}$ protein at 70 °C, pH 5.2 for various time intervals as described in Experimental Procedures, the DNA was deproteinized and introduced into *E. coli* by electroporation to score Kan^R revertants. Reversion frequencies with DNA incubated without protein (open squares), and with protein (closed squares) are shown.

deamination. When SDS was included in the reaction at a concentration of 1% (w/v), the protective abilities of the protein were reduced substantially. SspC$^{\Delta 11-D13K}$ reduced the revertant frequency to 19% of the DNA incubated without any protein and including SDS in the reaction increased the frequency to 59% (data not shown). While this showed that denatured protein offered much less protection than the native protein, we consistently found that the protein did provide some protection in the presence of SDS. It is possible that SDS somehow reduces the rate of cytosine deamination and affects the revertant frequency. Consistent with this interpretation, SDS alone suppressed moderately the increase in Kan^R revertant frequency (data not shown).

Because cytosines in DNA are often methylated, we also wished to know whether α/β-type SASP can provide protection against deamination of 5meC in addition to C. To test this, pUP31 DNA was first methylated by M.*Eco*RII in vitro and then incubated at 70 °C, pH 5.2, for 6 h with or without SspC$^{\Delta 11-D13K}$. M.*Eco*RII methylates the second cytosine in the mutated codon of the defective *kan* (*18*), and the T·G mismatches resulting from the deamination of this 5-methylcytosine are subject to very short-patch (VSP) repair (*6*). For this reason, the 5meC to T deaminations in methylated pUP31 were quantified by transforming the DNA into an *ung*$^+$ strain defective in VSP repair and scoring Kan^R revertants. Incubation of methylated pUP31 for 6 h in the absence of protein caused a ~24-fold increase in revertant frequency, but including the SspC$^{\Delta 11-D13K}$ protein in the reaction largely eliminated this increase (Figure 2). It is clear from these data that native SspC$^{\Delta 11-D13K}$ can protect both C's and 5meC's in DNA against deamination at acidic pH and high temperature.

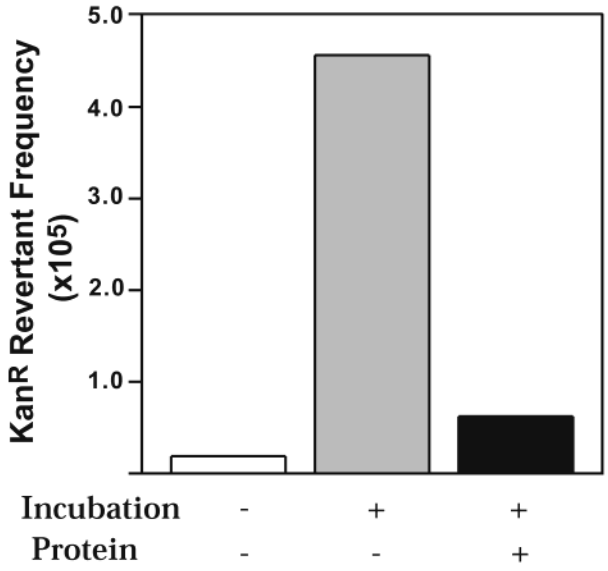

FIGURE 2: Protection of 5-methylcytosines by SspC$^{\Delta 11-D13K}$. Following incubation of methylated pUP31 DNA in the presence or absence of SspC$^{\Delta 11-D13K}$ protein at 70 °C, pH 5.2 for 6 h, the DNA was introduced into *E. coli* to score Kan^R revertants.

Protection of DNA by Other SspC Variants. We investigated the relationship between the ability of SspC to bind DNA and protection against cytosine deamination by studying the protection conferred by another variant, SspC$^{\Delta 11}$, and the wild-type protein at two different temperatures. The latter two proteins have different DNA-binding abilities than SspC$^{\Delta 11-D13K}$ (*20*). and we reasoned that this would affect their ability to protect DNA against cytosine deamination.

When the abilities of these three proteins to prevent cytosine deamination at 70 °C, pH 7.9 (at ambient temperature; predicted pH of this Tris-based buffer at 70 °C is 6.6). were compared, SspC$^{\Delta 11-D13K}$ performed substantially better than the other two proteins. While SspC$^{\Delta 11-D13K}$ reduced C to T mutations by a factor of 5, the other proteins had only a modest effect on the revertant frequency (Figure 3A). As roughly equal amounts of each of the three proteins were added to the reactions, these results show that the protection afforded by SspC$^{\Delta 11-D13K}$ against cytosine deamination was not somehow the consequence of high protein concentration in the reactions.

Incubation of plasmid DNA in the absence of any protein at 45 °C (predicted pH, 7.3) for 30 h resulted in only a 3.3-fold increase in revertants. This increase was largely eliminated when any of the three proteins was included in the reaction (Figure 4A). Although there was some variation in the magnitude of protection by the different proteins in different experiments, we consistently observed that WT SspC performed somewhat better than the other proteins (Figure 4A and data not shown).

Protection of Plasmid DNA against Restriction Digestion. In parallel experiments, we also assessed the binding of these proteins to DNA by studying their ability to protect the same plasmid DNA against cleavage by restriction enzymes. The DNA was digested with *Bst*DI (70 °C) or *Bst*NI (45 °C) in the same buffer used for the deamination studies in the presence or absence of the SspC proteins, and the products were separated by gel electrophoresis.

At both temperatures, the pattern of protection against restriction enzymes was generally consistent with the pattern

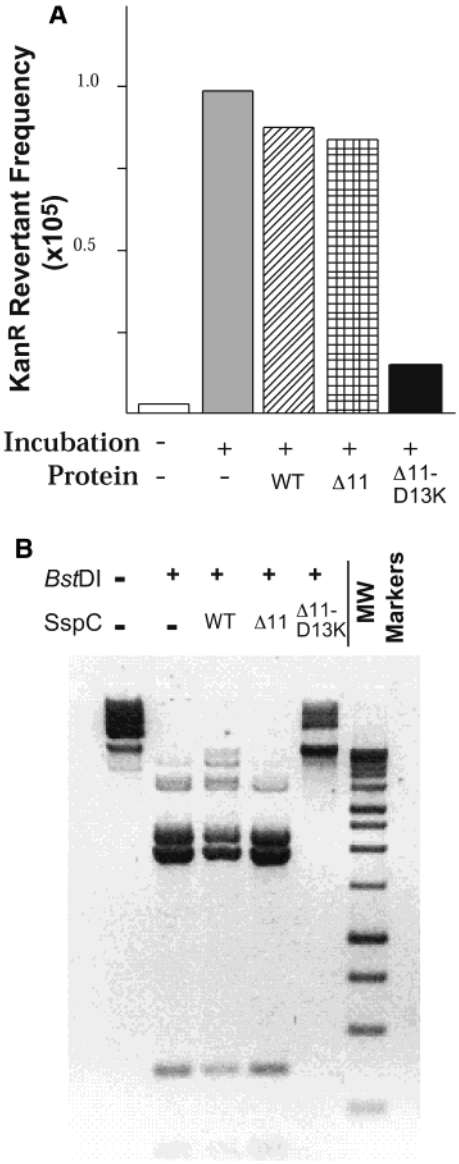

FIGURE 3: Comparison of protection by different SspC variants at 70 °C, pH 7.9. (A) Protection against cytosine deamination. Following incubation of pUP31 DNA with wild-type SspC and its variants for 6 h as described in Methods, the DNA was deproteinized and electroporated into *E. coli* to score Kan^R revertants. (B) Protection against digestion by *Bst*DI. PUP31 DNA was incubated with wild-type SspC and its variants for 3 h as described in Experimental Procedures and challenged with *Bst*DI for 30 min. The resulting fragments and unincubated control DNA were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed. The rightmost lane contains the kilobase ladder from Promega Corp. (Madison, WI).

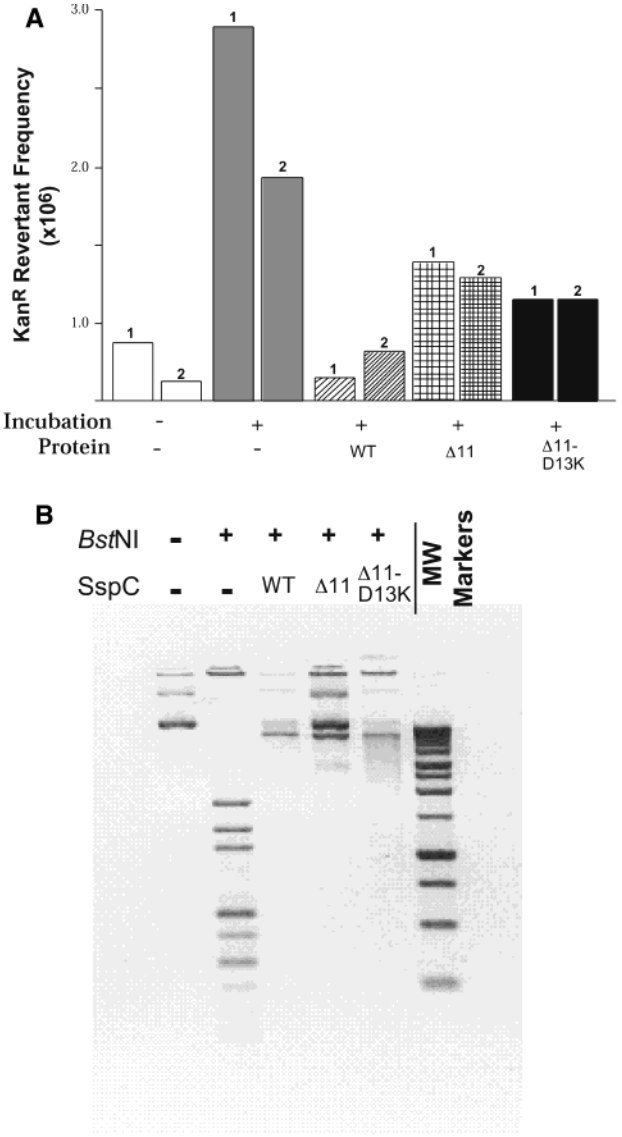

FIGURE 4: Comparison of protection by different SspC variants at 45 °C, pH 7.9. (A) Protection against cytosine deamination. Following incubation of pUP31 DNA with wild-type SspC and its variants for 30 h as described in Methods, the DNA was deproteinized and electroporated into *E. coli* to score Kan^R revertants. Two complete sets of data are shown and each bar in the graph is labeled with a number to identify the set to which it belongs. (B) Protection against digestion by *Bst*NI. PUP31 DNA was incubated with wild-type SspC and its variants for 18 h as described in Experimental Procedures and challenged with *Bst*NI for 30 min. The resulting fragments and unincubated control DNA were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed. The rightmost lane contains the kilobase ladder from Promega Corp. (Madison, WI).

of protection against cytosine deamination. At 70 °C, only $SspC^{\Delta 11-D13K}$ provided complete protection against *Bst*DI (Figure 3B). The DNA was largely susceptible to cleavage by *Bst*DI in the presence of either WT SspC or its Δ11 mutant in the reaction (Figure 3B). In contrast, all three proteins provided almost complete protection against *Bst*NI at 45 °C (Figure 4B). These data are consistent with the idea that it is the binding of these proteins to plasmid DNA that is responsible for the prevention of cytosine deamination.

DISCUSSION

We have shown here that SspC, a minor α/β-type SASP from *B. subtilis* , and its two of its N-terminal variants protect plasmid DNA against cytosine deamination at 45 °C, pH 7.9. It is expected that other SASPs including the major α/β-type spore proteins will also protect DNA in a similar manner. In addition, we found that one of the SspC variants protects DNA at 70 °C and at two different pH values. This latter protein also protects methylated cytosines in DNA against deamination to thymine. Further, there is a general correlation between the binding of these proteins to DNA and their ability to protect it against cytosine deamination. The results presented here show that the protective effects of these proteins are not a simple consequence of high protein concentration in the reaction mixtures somehow excluding

water from the vicinity of DNA, but a result of binding of the proteins to DNA. This suggests that other DNA-binding proteins may also protect DNA bases against attack by endogenous and exogenous chemicals and provides a novel mechanism by which cells may protect their genetic material.

In our initial experiments, we chose high temperature and low pH value (predicted pH 6.6) for the incubations to increase the sensitivity of the assay. Subsequently, we found that our assay was sensitive enough to detect a reduction in the modest amount of deamination that occurs over a 30 h period during incubation at 45 °C, pH 7.3 (predicted). Therefore, it is very likely that this form of DNA base protection can be effective at physiological pH and ambient temperature, i.e., under conditions that exist within cells and *Bacillus* spores (*22*). Indeed, there is no mutagenesis of wild-type *B. subtilis* spores incubated for long periods at elevated temperatures, while spores lacking the majority of their α/*β*-type SASP are mutagenized with high efficiency by these same temperatures (*13*). In particular, the most frequent spontaneous base substitutions in spores are C:G to T:A transitions, and the frequency of these mutations is 4-fold higher in spores lacking α/*β* proteins (*23*) The work presented here suggests that one of the reasons for the increase in mutations in spores lacking α/*β*-type SASP is due to attack of DNA bases by water. It is useful to note that the α/*β*-type SASP also provide protection against hydrolytic cleavage of the glycosylic bond in DNA in vivo as well as in vitro (*13*).

Cytosines in single-stranded DNA are substantially more susceptible to hydrolytic deamination than in double-stranded DNA (*24*, *25*). Consequently, SspC may protect cytosines in DNA by preventing "breathing" of DNA. However, two other aspects of the *Bacillus* spore structure may also be relevant to the prevention of cytosine deamination in DNA. The spore core, the site of spore DNA, contains substantially less water than a vegetative cell; indeed, the resistance of the spores to wet heat is inversely related to the spore core's water content (*26*). While the spore cortex is thought to be involved in reducing the core water content (*27*), it is possible that tight cooperative binding of spore DNA by proteins such as α/*β*-type SASPs further removes free water from the immediate vicinity of DNA, and this provides protection against hydrolytic attack on the DNA bases.

A second aspect of the α/*β*-type SASP-DNA interactions that may be relevant to our study is that the binding of α/*β*-type SASP to DNA changes the latter to an A-like helix (*28*). It is possible that A-form DNA is more resistant to cytosine deamination than the B-form. However, no data exist regarding the rates of deamination of cytosines in different forms of DNA, and hence this is currently only a speculation.

The DNA in cells is rarely free of proteins. The ubiquitous DNA-binding proteins in bacterial cells include HU and H/NS (*29*), while in eukaryotes histones, methyl-CpG binding proteins (*30*) and other transcription-related proteins coat the genome. It is known that nucleosomes not only protect DNA against endonucleases and exogenous chemicals, they also protect it against attack by hydroxyl radicals (*31*). Therefore, it is reasonable to think that nucleosomes and other DNA-binding proteins may provide some protection for DNA against water attack. In fact, a role for DNA-binding proteins in protecting DNA has been suggested in hyperthermophiles (*32*), but no evidence has been presented to support the hypothesis. Even during the processes of replication and transcription when DNA strands must be separated, some proteins remain associated with the DNA strands. During replication, the exposed single stranded regions are coated with SS DNA-binding proteins (Ssb in bacteria). It is known that Ssb can protect DNA against attack by the chemical mutagen bisulfite (*33*), and it is likely that this protein protects SS DNA also against attack by water and other endogenous chemicals.

It should be noted that not all DNA-binding proteins may protect DNA. 5-Hydroxymethyl cytosines in bacteriophage T4 genome within phage particles suffer deaminations at a rate that is not suggtestive of DNA protection by phage proteins (*36*). Cytosine methyltransferases are a class of enzymes that deaminate cytosines (*18*, *34*) and 5-methyl-cytosines in DNA (*35*). Additionally, proteins that unwind DNA such as helicases or that kink and distort DNA such as transcription factors, may expose some DNA bases to chemical agents. Thus, the question of whether DNA-binding protein protects cytosines in DNA or not should be evaluated on a case-by-case basis.

In summary, we have shown here that a DNA-binding protein from *Bacillus* spores provides protection of DNA against the most commonly found DNA-damaging agent, water. It is likely that it does so by excluding free water from the vicinity of DNA and other cellular DNA-binding proteins may also provide this form of protection to DNA.

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