

Accepted Manuscript

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Authors: Linlin Yang, Yajun Jian, Peter Setlow, Lei Li



PII: S1568-7864(15)30118-X
DOI: <http://dx.doi.org/doi:10.1016/j.dnarep.2016.11.005>
Reference: DNAREP 2348

To appear in: *DNA Repair*

Received date: 1-12-2015
Revised date: 23-5-2016
Accepted date: 15-11-2016

Please cite this article as: Linlin Yang, Yajun Jian, Peter Setlow, Lei Li, Spore photoproduct within DNA is a surprisingly poor substrate for its designated repair enzyme—the spore photoproduct lyase, *DNA Repair* <http://dx.doi.org/10.1016/j.dnarep.2016.11.005>

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Spore photoproduct within DNA is a surprisingly poor substrate for its designated repair enzyme – the spore photoproduct lyase

Linlin Yang,[†] Yajun Jian,[†] Peter Setlow[⊥] and Lei Li^{†‡*}

[†]Department of Chemistry and Chemical Biology, Indiana University-Purdue University Indianapolis (IUPUI), 402 North Blackford Street, Indianapolis, Indiana, 46202

[‡]Department of Biochemistry and Molecular Biology & Department of Dermatology, Indiana University School of Medicine, Indianapolis, Indiana 46202

[⊥]Department of Molecular Biology and Biophysics, UConn Health, Farmington, Connecticut, 06030

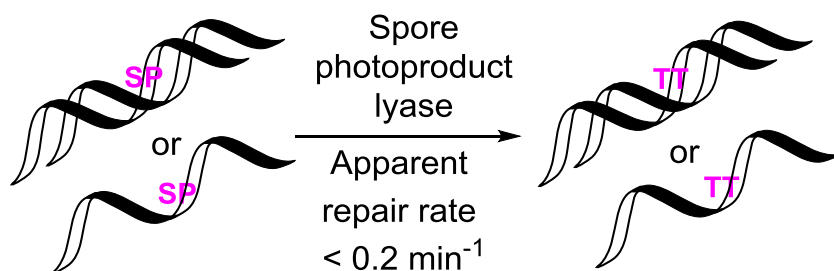
lilei@iupui.edu

Corresponding Author *Email: lilei@iupui.edu

Author Contributions L.Y. and Y.J. conducted the enzymology studies and oligonucleotide syntheses respectively. L.L and P.S. wrote the manuscript.

Notes The authors declare no competing financial interest.

Graphical abstract



ABSTRACT:

DNA repair enzymes typically recognize their substrate lesions with high affinity to ensure efficient lesion repair. In UV irradiated endospores, a special thymine dimer, 5-thyminyl-5,6-dihydrothymine, termed the spore photoproduct (SP), is the dominant DNA photolesion, which is rapidly repaired during spore outgrowth mainly by spore photoproduct lyase (SPL) using an unprecedented protein-harbored radical transfer process. Surprisingly, our *in vitro* studies using SP-containing short oligonucleotides, pUC 18 plasmid DNA, and *E. coli* genomic DNA found that they are all poor substrates for SPL in general, exhibiting turnover numbers of $0.01\text{-}0.2 \text{ min}^{-1}$. The faster turnover numbers are reached under single turnover conditions, and SPL activity is low with oligonucleotide substrates at higher concentrations. Moreover, SP-containing oligonucleotides do not go past one turnover. In contrast, the dinucleotide SP TpT exhibits a turnover number of $0.3 \sim 0.4 \text{ min}^{-1}$, and the reaction may reach up to 10 turnovers. These observations distinguish SPL from other specialized DNA repair enzymes. To the best of our knowledge, SPL represents an unprecedented example of a major DNA repair enzyme that cannot effectively repair its substrate lesion within the normal DNA conformation adopted in growing cells. Factors such as other DNA binding proteins, helicases or an altered DNA conformation may cooperate with SPL to enable efficient SP repair in germinating spores. Therefore, both SP formation and SP repair are likely to be tightly controlled by the unique cellular environment in dormant and outgrowing spore-forming bacteria, and thus SP repair may be extremely slow in non-spore-forming organisms.

INTRODUCTION

Spore photoproduct lyase (SPL) is a key DNA repair enzyme in outgrowing bacterial spores, catalyzing the repair of the 5-thymine-5,6-dihydrothymine lesion, commonly called the spore photoproduct (SP).¹⁻⁴ SP, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) (Figure 1) are the three major pyrimidine dimers formed under UV light in DNA. All these dimers can be repaired by a direct reversal mechanism, i.e. the dimer is reverted back to two pyrimidine residues by breaking the crosslinking bonds. Direct reversal repair of CPDs and 6-4PPs is performed by specific photolyases,⁵⁻¹⁰ which use light and flavin cofactors to generate radical species in the dimers before bond scission results in two pyrimidine residues. In contrast, SPL is a so-called radical SAM (*S*-adenosylmethionine) enzyme,¹¹⁻¹⁵ which initiates SP repair by reductively cleaving the SAM cofactor to a 5'-deoxyadenosyl (5'-dA) radical and methionine. Protein sequence analysis has identified a homologous region in the carboxyl-terminal portions of the CPD photolyases and SPL, indicating that these enzymes may have descended from a common ancestral protein.¹⁶ Photolyases repair CPDs/6-4PPs within a single- or double-stranded (ss/ds) oligonucleotide with much higher efficiency than those in the dinucleotide context due to higher substrate binding affinity to the enzyme, and it has been believed that SPL will exhibit a similar substrate preference.^{8,14,17,18}

SPL is suggested to adopt a radical mechanism to directly revert SP to two thymine residues.^{19,20} As shown in Figure 2, the currently accepted mechanism implies that SPL utilizes the 5'-dA radical to abstract the H_{6proR} atom to initiate the SP repair process.^{18,21} The resulting thymine allylic radical abstracts an H-atom from a conserved cysteine residue, C141 in *B. subtilis* (*Bs*) SPL, to produce the repaired thymine residues.^{17,22} Such a process leaves a thiol radical on the enzyme, which is hypothesized to abstract an H-atom from the neighboring Y99(*Bs*). The Y99(*Bs*) radical then oxidizes 5'-dA with the assistance of Y97, regenerating the 5'-dA radical before regenerating the SAM molecule.²³

The assumption that SAM is recycled in SPL catalysis was originally proposed based on an experiment

using SP-containing pUC 18 plasmid DNA as the enzyme substrate, which found that one SAM may catalyze > 500 turnovers with a turnover number of 12.4 min^{-1} .^{24,25} Other studies using smaller but chemically better-defined substrates, however, obtained much lower turnover numbers.^{17,18,23,26,27} For instance, using dinucleotide SP TpT as the substrate, various groups reported the SPL turnover number to be $\sim 0.3 \text{ min}^{-1}$.^{16,17,22,25} Moreover, SAM was at most partially regenerated in these studies. As the CPD/6-4PP repair studies show that photolyases exhibit much higher binding affinity toward pyrimidine dimers within single-/double-stranded (ss/ds) oligonucleotides than in the dinucleotide context, which consequently leads to much higher repair efficiency, the drastically different SPL activity and SAM regeneration patterns among these previous studies have been ascribed to the variously-sized SP substrates used, which result in various binding affinities toward SPL.

Using the SP phosphoramidites developed in our group,²⁸ we are now able to incorporate dinucleotide SP TpT or dinucleoside SP TT into oligonucleotides under well-controlled local environments. Also, using DNA photoreaction in ice,²⁹ it is possible to prepare SP-containing plasmid or genomic DNA. The SP contents in these polymers can be quantified by mass spectrometry after DNA digestion by enzymes using multiple reaction monitoring coupled with stable isotope dilution mass spectrometry (SID-MRM-MS).³⁰ We therefore re-examined SPL activity using SP substrates under a wide range of sequence environments. Surprisingly, the results indicate that SPs within an oligonucleotide are much worse SPL substrates than the dinucleotide SP TpT. More importantly, the resulting repair efficiency of SP in oligonucleotides appears to be too low to account for SP repair in outgrowing spores. In addition, SPL enzyme activity is drastically reduced in the presence of an excess of SP-containing oligonucleotides, suggesting the occurrence of possible substrate inhibition. The enzyme also does not go beyond a single turnover when SP TpT-containing oligonucleotides are used, indicating that product release may be slow. Our data suggest that SP represents an unprecedented example of a major DNA lesion within a normal genomic DNA environment that is difficult to be recognized and repaired by its designated repair enzyme. Consequently, other factors in germinating spores may have to assist SPL-mediated SP repair.

METHODS

Materials. DNA-modifying enzymes were purchased from Fermentas Life Sciences (Glen Burnie, MD). Oligonucleotides without an SP lesion were synthesized by Integrated DNA Technologies (Coralville, IA). *E. coli* BL21 (DE3) and the expression vector pET-28a were purchased from Novagen (Madison, WI). The pUC 18 plasmid was purchased from Thermo Fisher Scientific Inc. The phosphoramidites for A, T, C and G were purchased from Glen Research (Sterling, VA). All other buffers and chemicals were of the highest grade commercially available from Fisher, VWR or Sigma-Aldrich. *B. subtilis* SPL was cloned, expressed and purified as previously described.^{17,18}

Preparation of SP substrates. The dinucleotide SP TpT was synthesized as previously described.³¹ The *d*₃-SP was photochemically synthesized using dinucleotide *d*₃-TpT containing a –CD₃ moiety at the 3'-thymine in a dry film reaction.³¹ The phosphoramidites for dinucleotide SP TpT and dinucleoside SP TT were prepared using our established procedure; syntheses of the SP-containing 10-mer and 20-mer oligonucleotides were conducted as described previously.²⁸ The 13-mer oligonucleotide containing SP TT was prepared using SP TT phosphoramidite and an ABI 3400 DNA/RNA synthesizer; the characterization of the resulting 13-nucleotide is shown in the Supporting Information. The sequences of the oligonucleotides used in this study are listed in Table 1.

The SP-containing *E. coli* genomic DNA or pUC 18 plasmid was prepared via UV irradiation in ice at –78 °C using a Spectroline germicidal UV sterilizing lamp (Dual-tube, 15 w, intensity: 1550 uw/cm², Spectronics Corporation, Westbury, NY) with the samples ~5 cm from the lamp.³¹ The lamp emits unfiltered UVC radiation centered at 254 nm. To quantify the SPs formed, the resulting genomic DNA was digested into a mixture containing single nucleosides, dinucleotide SP TpT, and other lesions using a combination of calf-spleen phosphodiesterase, snake-venom phosphodiesterase, nuclease P1, and alkaline phosphatase following literature protocols.³²⁻³⁴ A known amount of *d*₃-SP TpT was then added to the digest as an internal standard. The digest was analyzed by LC-MS using an Agilent 1200-6410 LC/MS triple quadrupole mass spectrometer as described below and the SP was quantified by comparing the

signal intensities from the endogenous unlabeled SP TpT with the exogenous labeled d_3 -SP TpT (Figure 3). It is worth pointing out that unlabeled molecules may behave slightly differently in chromatography from their deuterated isotopologues,³⁰ leading to differential suppression or enhancement of ionization. Such a phenomenon was observed for d_0 -/ d_3 -SP TpT, as reflected by the slope of 0.9 found in the calibration curve (Figure 3D). Using the calibration curve to assist product quantification, we found that a 1-hr UV irradiation converted ~2% of total Ts in pUC 18 plasmid into SPs, the yield of which is similar to that reported previously.²⁴ Under similar conditions, ~8% of total Ts in *E. coli* genomic DNA were converted into SPs.

SPL activity assay SPL activity was analyzed as previously described.²³ Typically, a reaction mixture contained 2 nmole $SPL_{(Bs)}$, 20 nmole SP TpT substrate, and 20 nmole SAM in 400 μ L buffer containing 25 mM Tris-HCl, 250 mM NaCl and 10% glycerol at pH 7.0. These components were incubated for 30 minutes under an inert atmosphere at ambient temperature to allow SPL enough time to find its substrate before 200 nmole sodium dithionite was added to reduce the SPL [4Fe-4S] cluster and initiate the enzyme reaction. The reaction was carried out under anaerobic conditions at ambient temperature for various periods of time. At each time point, 40 μ L of the solution was aliquoted to an Eppendorf tube containing 80 μ L ice-cold water. The solution was then kept in a -20 °C coldbox until analysis. Such a quenching method results in the same SPL activity as that obtained previously via acid quenching,^{17,18,23} proving the effectiveness of the method. After removing protein via extraction with phenol/chloroform, the supernatant fluids were loaded onto HPLC, separated, and analyzed using procedures described below.

The enzyme reactions using synthesized SP TpT or SP TT-containing 10-mer, 13-mer and 20-mer ss/ds oligonucleotides were conducted using the assay conditions described above containing 1 nmole SPL, 1~5 nmole oligonucleotides and 5 ~ 10 nmole SAM. After the 30-minute incubation, the reactions again were initiated by addition of 200 nmole sodium dithionite, stopped by addition of ice water, protein removed, and HPLC analysis carried out.

When SP-containing pUC 18 plasmid or *E. coli* genomic DNA was used as the substrate, the reaction solutions contained 1 nmole SPL, 0.16 ~ 20 nmole SP, and 5 ~ 10 nmole SAM. The reactions were stopped by ice water addition at various times as described above. The resulting mixtures were later exposed to O₂ to ensure complete inactivation of the SPL before enzyme digestion of DNA.³²⁻³⁴ A known amount of *d*₃-SP TpT was added as the internal standard to help quantify the unreacted SP TpT left in the reaction by LC-MS.

HPLC assay HPLC was performed with detection at 260 nm using a Shimadzu LC-20AB high-pressure gradient solvent delivery unit coupled with a SPD-20A UV-Vis detector and a CTO-10AS VP Column Oven. A Waters XBridgeTM OST reverse-phase C18 column (2.5 μm, 4.6 × 50 mm) was used for all separation work.

Analysis of dinucleotide SP TpT repair was conducted using our previously described HPLC procedure where 50 mM triethylammonium acetate (TEAA), pH 6.5, was used as Mobile Phase A and compounds were eluted with an ascending gradient (0 – 25%) of Mobile Phase B which was composed of 50% Mobile Phase A and 50% acetonitrile at a flow rate of 1 mL/min.^{14,17,18}

For analysis of SP repair in an oligonucleotide, 10 mM TEAA at pH 7.0 was used as Mobile Phase A, and 10 mM TEAA in 67% water and 33% acetonitrile at pH 7.0 was used as Mobile Phase B. The flow rate was 1 mL/min. To analyze SP TpT repair in the ss 10-mer d(CACC [SP TpT] CATC), the chromatography was performed at ambient temperature, beginning with 8% Mobile Phase B followed by an ascending gradient of Mobile Phase B at 1% per minute. With this gradient, 5'-dA was eluted at 6.8 min, the SP TpT-containing 10-mer at 13.9 min and the repaired 10-mer d(CACCTTCATC) at 16.4 min. To analyze SP TT repair in the ss 13-mer d(CGTGA [SP TT] ACAGCC), HPLC was again performed at ambient temperature, starting with 100% Mobile Phase A for 1 min followed by an ascending gradient of Mobile Phase B at 2% per min for 8 min. The gradient was then changed to 0.3% for the next 10 min followed by 1% for another 8 min. With this gradient, 5'-dA was eluted at 9.1 min, the 6-mer d(CGTGAT) and 7-mer d(TACAGCC) resulting from the repair of the SP TT in the 13-mer were eluted at 14.3 min and 15.3 min respectively, and the SP TT containing 13-mer eluted at 21.2 min. The same program can

also be used to analyze SP TT repair reactions in the duplex context, where the complementary strand d(GGCTGTAATCACG) was eluted at 22.6 min.

To analyze SP TT repair in the 20-mer d(CTCGACACG[SP TT]CGCATGCCA) in either a ss or ds context, HPLC was conducted with the column heated to 60 °C in an oven to ensure complete separation of the two strands in the duplex. We started with 100% Mobile Phase A for 1 min followed by an ascending gradient of Mobile Phase B at 2% per min for 8 min and then 0.5% for 10 min. With this gradient, 5'-dA was eluted at 7.3 min, the two 10-mer fragments d(CTCGACACGT) and d(TCGCATGCCA) resulting from the repair of the SP TT in the 20-mer were eluted at 11.2 min and 11.9 min respectively, and the SP TT containing 20-mer was eluted at 17.9 min. The same program can be used to analyze SP TT repair reactions in the ds context, where the complementary strand d(TGGCATGCGAACGTGTCGAG) was eluted at 13.4 min. The program was also used to analyze SP TpT repair in the 20-mer oligonucleotide with the same sequence in either a ss or ds context. The SP TpT containing 20-mer d(CTCGACACG[SP TpT]CGCATGCCA) was eluted at 14.5 min and the repaired 20-mer eluted at 16.5 min.

SID-LC-MRM-MS Analysis Multiple reaction monitoring coupled with stable isotope dilution mass spectrometry (SID-MRM-MS) has proven to be a powerful method for quantitative measurement of nucleic acid and protein biomarkers.³⁰ SP repair by SPL in pUC 18 plasmid or *E. coli* genomic DNA was analyzed via an Agilent 1200-6410 LC-MS triple quadrupole mass spectrometer under the MRM mode. Enzyme digestion of pUC 18 plasmid or *E. coli* genomic DNA releases the endogenous unlabeled dinucleotide SP TpT, which was then quantified via mass spectrometry by comparing its signal intensity with that from a known amount of added *d*₃-SP TpT using literature protocols (Figure 3).³⁵⁻³⁷

The HPLC experiments were conducted using a ZORBAX Eclipse plus C18 column 3.0 × 100 mm (1.8 μm in particle size, Agilent Technologies, Santa Clara, CA) at 55 °C with a flow rate of 200 μL/min. 0.1% (v/v) formic acid in water (Mobile Phase A) and a solution of 0.1% (v/v) formic acid in acetonitrile (Mobile Phase B) were used as mobile phases for the 5'-dA analyses. A solution of 5 mM ammonium acetate in water (Mobile Phase A) and a solution of 5 mM ammonium acetate in 1 : 1 methanol/acetonitrile (Mobile Phase B) were used for SP TpT analysis. An isocratic flow of 5% Mobile Phase B was used for

the separation and analysis of 5'-dA. To analyze SP TpT, we started with 5% Mobile Phase B for 1 min followed by a gradient of 1% per min for 25 minutes. With this gradient, SP TpT was eluted at 7.3 min and TpT was eluted at 20.5 min.

The eluted compounds were introduced into the Agilent 6410 triple quadrupole mass spectrometer. The temperature for the sheath gas flow was maintained at 350 °C, the gas flow rate was 11 L/min, and the capillary voltage was 4 kV. The fragmentor voltage was optimized to 225 V for SP TpT, *d*₃-SP TpT and TpT analyses and to 110 V for 5'-dA analysis. We selected the parent ions of SP TpT and TpT (*m/z* 545.2) as well as *d*₃-SP TpT (*m/z* 548.2) and monitored fragments with *m/z* of 251.1 (34V), 195.0 (34 V) and 78.9 (82 V) for SP TpT, *m/z* of 254.1 (34 V), 195.0 (34 V) and 78.9 (82 V) for *d*₃-SP TpT and *m/z* of 195.0 (34 V) and 125.0 (34 V) for TpT under the negative mode (Figure 3). We selected the parent ion of 5'-dA (*m/z* 252.2) and monitored the fragment with *m/z* of 118.5 (52 V) under the positive ion mode. The collision energy for each fragment was optimized and is listed in the parenthesis next to the fragment above. Linear responses between the intensity of the mass spectrometric signals and the amount of the analytes were observed, demonstrating the feasibility of using this LC-MRM-MS assay for quantitative analysis. Therefore, for the 5'-dA and TpT generated in the SPL reaction, their amounts were readily quantified by calculating from the peak area found in the selected-ion chromatograms for the analytes. Since only a small portion of SP TpT was repaired by SPL, it was essential to determine the amount of SP remaining at a given time with a high accuracy in order to calculate enzyme activity. The *d*₃-SP TpT was thus added to the DNA digest as an internal standard. The amount of endogenous unlabeled SP TpT in the nucleoside mixtures was calculated from the ratios of areas of peaks found in the selected-ion chromatograms for the analyte over the *d*₃-SP TpT standard and the amount of *d*₃-SP TpT added and the calibration curve shown in Figure 3.

RESULTS

SP TpT repair in a ss 10-mer As CPD photolyase binds to CPDs in ss and ds DNA with the same

affinity,⁸ we first investigated SP TpT repair in the ss 10-mer d(CACC[SP TpT]CATC). By DNase I footprinting, SPL was shown to protect an SP-containing 9-nt region, with five nt at the 5'-side and two nt at the 3'-side of the SP.³⁹ Most of this binding region was included in the 10-mer sequence studied in the current work. With the HPLC procedure used, the SP-containing 10-mer and repaired 10-mer elute at 13.9 min and 16.4 min respectively (Figure 4), making product quantitation straightforward via HPLC peak integration. Surprisingly, using an HPLC calibration curve prepared using authentic d(CACCTTCATC), the WT SPL repaired SP in this 10-mer at only 0.007 min⁻¹ (Table 2). In contrast, the same WT SPL was found to repair the dinucleotide SP TpT at 0.32 min⁻¹, a value comparable to that reported for this substrate previously,^{18,26} but ~40-fold faster than SP TpT repair in the ss 10-mer. Moreover, the ratio between repaired SP and 5'-dA generated was 1.4 : 1, comparable to the ratio of 1.5 : 1 obtained from the SPL reaction using SP TpT as the substrate.¹⁸

SP TT repair in a ss 13-mer Recently, by using a ss 13-mer sequence containing a dinucleoside SP TT (Figure 1) whose concentration is 5-fold higher than that of SPL from *Geobacillus thermodenitrificans*, Benjdia et al. reported an SP repair reaction rate of 1.66 nmol min⁻¹ mg⁻¹ (~ 0.066 min⁻¹).⁴⁰ We therefore synthesized a 13-mer d(CGTGA[SP TT]ACAGCC) using the SP TT phosphoramidite,²⁸ and studied its repair by SPL. As shown in Figure 5, the repair of SP TT results in a 6-mer and a 7-mer fragment; the repair efficiency by our SPL enzyme was found to be ~ 0.08 min⁻¹ when the level of the 13-mer substrate was 2-fold higher than that of SPL. Moreover, using a ss SP TT-containing 20 mer sequence with a similar substrate concentration, a turnover number of 0.09 min⁻¹ was obtained. Interestingly, increasing the oligonucleotide concentration up to 5-fold that of SPL reduced the enzyme activity ~ 3 fold (Table 2). These activities are similar to what Benjdia et al. determined previously, but are ~ 10 times faster than that determined with a ss SP TpT-containing 10-mer oligonucleotide. Even so, the slow repair rate and the gradual loss of enzyme activity led to only 1 - 2 turnovers after an overnight reaction, contrasting with the 10 turnovers supported by dinucleotide SP TpT

under similar reaction conditions.^{17,18} Additionally, the ratio between the amount of SP TT repaired and the 5'-dA generated was close to 1 using both oligonucleotides, suggesting that SAM was barely regenerated. Furthermore, the presence of excess substrate worsened the SAM regeneration process, as suggested by the ratio of repaired SP/ 5'-dA generated using 5-fold higher levels of the SP TT-containing 20-mer.

SP TT repair in the ds 13-mer After investigating SP repair in ss oligonucleotides, we next examined repair in ds oligonucleotides. We first studied SP TT repair in the 13-mer sequence at a concentration twice that of the enzyme. Product analysis revealed that the SP TT repair in this duplex environment occurs at $\sim 0.04 \text{ min}^{-1}$, which is roughly half of the rate observed in the ss 13-mer under similar conditions. Again, no significant 5'-dA regeneration was noted (Table 2).

SP TT repair in a ds 20-mer In 10 mM phosphate buffer containing 250 mM NaCl at pH 7.0, the melting point (T_m) of the ds SP TT-containing 13-mer was found to be 39 °C at 2 μM concentration. The melting curve indicates that at the ambient temperature (24 °C) under which the SPL reaction was conducted, only $\sim 70\%$ of the SP TT-containing strand would be in the duplex form. To confirm that the SPL activity observed was due to substrate in the duplex oligomer, we studied SP TT repair in the ds 20-mer d(CTCGACACG[SP TT]CGCATGCCA)/d(TGGCATGCGAACGTGTCGAG). This duplex exhibits a T_m of 64.6 °C,²⁸ indicating that $> 99\%$ of SP TT should be in the duplex form at 24°C. The HPLC chromatography was conducted at 60 °C to ensure a good separation of the two strands in this ds 20-mer. Repair of SP TT generates two 10-mer strands (Figure 6A), which can be quantified after HPLC separation. Examination of SPL activity reveals that SP was repaired at $0.03 \pm 0.01 \text{ min}^{-1}$ when the concentrations of SP-containing oligonucleotide and enzyme were equivalent, which is comparable to the repair rate with the 13-mer ds oligonucleotides. However, when 5-fold more of the SP-containing duplex strand relative to the enzyme was added, the repair rate was reduced to $\sim 0.01 \text{ min}^{-1}$ (Table 2). Under

either concentration tested, the overnight SP repair reaction only finished 0.6 ~ 0.7 turnovers. Interestingly, at the lower substrate concentration (SP/SPL=1 : 2), the ratio between SP repaired / 5'-dA generated was close to 1; the number decreased to 0.5 at a higher substrate concentration. Such observations imply that the higher substrate concentrations not only slow down SP repair, but also interfere with SAM regeneration.

SP TpT repair in a ds 20-mer We next examined SP TpT repair in the ds 20-mer with the same sequence used above. Again, product analysis by HPLC was conducted at 60 °C, and good separation was achieved among the SP-containing ss 20-mer, the repaired strand and the complementary strand (Figure 6B). Analysis of the reaction revealed that the SP TpT repair rate was almost identical to SP TT repair in the ds 20-mer. As summarized in Table 2, these two reactions share almost identical results in terms of the SP repair rate, the overall turnover number, and the ratio between SP repaired and 5'-dA generated at a given substrate concentration.

LC-MRM-MS analysis of SP TpT repair As noted above, SP repair in short ds oligonucleotides was very slow. To ensure that the slow rate is not an artifact resulting from inactive enzymes, we then re-examined SPL reaction using the dinucleotide SP TpT as the substrate. Using the HPLC assay established in our previous studies to analyze TpT formation,^{17,18,23} a turnover number of ~ 0.32 min⁻¹ was obtained. Moreover, via mass spectrometry under MRM mode, the TpT generated in this assay was also quantified by integration of the MS signals after HPLC separation and comparison with a calibration curve constructed with known amounts of authentic samples. Plotting the amount of TpT generated in this assay against reaction time, also gave a TpT formation rate of 0.3 ± 0.03 min⁻¹.

As SP repair proceeds, the amount of SP in the reaction would gradually decrease. Plotting the reduction of SP against time also allows us to calculate the SP repair rate. To minimize random errors during SP quantification, a known amount of *d*₃-SP TpT was added as an internal standard before the MRM-MS analysis. The amount of unlabeled SP TpT left in the reaction solution can be accurately

determined by comparing its signal intensity with that from the d_3 -SP TpT by MS, and this gave an SP repair rate by SPL of $0.31 \pm 0.03 \text{ min}^{-1}$. The identical TpT formation and SP TpT disappearance rates obtained demonstrate the validity of using SP TpT disappearance as a marker to quantify SPL activity.

The 5'-dA found was also quantitated using the LC-MRM-MS assay under the positive ion mode. This MS assay is at least 100-fold more sensitive than the assay using HPLC alone, making 5'-dA quantitation feasible even when its concentration is very low. Using the LC-MRM-MS assay, the ratio between SP repaired (TpT formed)/ 5'-dA generated was 1.5 ± 0.2 , indicating only partial SAM regeneration (Table 2). All these results agree with those obtained in our previous studies,^{17,18,23} as well as those conducted by other groups,^{26,41,42} proving that the enzyme used is fully active.

SP TpT repair in pUC 18 plasmid DNA The SID-LC-MRM-MS analysis of dinucleotide SP TpT repair demonstrated the effectiveness of the assay, and we then utilized it to analyze SP repair by SPL in pUC 18 plasmid DNA. After enzyme treatment using a combination of calf-spleen phosphodiesterase, snake-venom phosphodiesterase, nuclease P1, and alkaline phosphatase, the genomic DNA was digested into the mononucleosides A, T, C and G while SP is released as the dinucleotide SP TpT.³² These small molecules can be readily separated by HPLC and the SP eluted is a mixture of endogenous unlabeled SP TpT and its exogenous isotopologue d_3 -SP TpT (Figure 3C). Using d_3 -SP TpT as the standard, the amount of unlabeled SP TpT in a sample can be readily quantified.

Using our SID-LC-MRM-MS assay, we found that SPL repairs SP in pUC 18 plasmid very slowly, $\sim 0.05 \text{ min}^{-1}$ when the added SP roughly equaled that of the enzyme (Table 2), which is slightly faster than SP repair in the ds 20-mer. The reaction slowed drastically after 5 minutes; an overnight reaction only resulted in ~ 0.3 turnover, which is half of that observed from SP repair in the ds 20-mer. Moreover, when plasmid containing 10-fold more SP than the SPL added, the enzyme activity became so low that it could not be reliably determined. Such a trend of enzyme activity reduction with an increasing amount of SP substrate is consistent with our studies using short ss- and ds- SP-containing oligonucleotides as substrates, and is opposite to the results found in a previous study.^{24,25}

SP TpT repair in *E. coli* genomic DNA To further confirm our results, we then examined SP repair in *E. coli* genomic DNA using our SID-LC-MRM-MS assay. Again SP repair by SPL was drastically affected by the amount of SP substrate added. When the SP concentration equaled that of the enzyme, the SP repair rate was $\sim 0.06 \text{ min}^{-1}$ (Table 2), comparable to the SP repair rate supported by pUC 18 plasmid or the ds 20-mer. However, when SPs added in the genomic DNA were > 10 equivalents of the SPL used, the enzyme activity was too low to be determined. On the other hand, when the added genomic DNA contained SPs that were 1/6 of the SPL used, the SP repair by SPL was much faster, as the enzyme exhibited a turnover number of 0.23 min^{-1} assuming that only 1/6 of the added enzyme interacted with a substrate and thus participated in SP repair. However, in none of these cases did a prolonged enzyme reaction finish one turnover.

Although the SP repair rate and the overall turnover number with SP-containing pUC 18 plasmid or *E. coli* genomic DNA were consistent with those with the SP-containing ds 20-mer, much less 5'-dA was generated in the plasmid or genomic DNA reactions. Quantification of 5'-dA by our LC-MRM-MS assay under the positive ion mode found that when 6-fold higher levels of SPL were present, repair of every ~ 8 SP TpT molecules in genomic DNA generated one 5'-dA (Table 2), indicating that SAM was truly regenerated. Even when equivalent amounts of SPL and SP were present, the repaired SP was still 3 times more than the 5'-dA generated. Such observations were in sharp contrast to those observed in repair reactions with SP-containing short oligonucleotides, indicating that besides the SPL-SP interaction at the substrate binding pocket, the longer plasmid or genomic DNA may have some extra interactions with SPL that may restrict the enzyme to the “correct” conformation, enabling SAM regeneration.

DISCUSSION

SPL activity with various substrates SPL catalyzes the repair of SP in outgrowing spores. The *B. subtilis* genome has 4.2 million base pairs (bp),⁴³ and high UV doses can convert up to 28% of thymine

residues in the spore genome to SP,⁴⁴ which may result in spore killing likely due to an overwhelmed SP repair system.⁴⁵ Previous work has shown that >75% of SPs are repaired during the first hour of spore germination and outgrowth.⁴⁶ Each spore may contain up to 200 molecules of SPL.⁴⁷ If 6% of total Ts are converted to SPs and only half of these SPs are repaired by SPL and the other half by the nucleotide excision repair (NER) pathway,^{48,49} each SPL needs to repair ~ 4 SPs per minute in outgrowing spores. Given the large size of the bacterial genome, SPL may take some time to find an SP lesion.⁵⁰ Thus, the actual SPL activity *in vivo* may be even higher.

Surprisingly, the prediction of SPL activity *in vivo* given above is not consistent with our *in vitro* SPL studies reported here using SP-containing ss and ds oligonucleotides as substrates. All substrates exhibit low SPL activity at $< 0.2 \text{ min}^{-1}$. The order of the SP repair efficiency was found to be: SP TpT in ss oligomer $<$ SP TT in ds oligomer \approx SP TpT in ds oligomer \approx SP TpT in pUC 18 plasmid or *E. coli* genomic DNA $<$ SP TT in ss oligomer $<$ dinucleotide SP TpT, which is totally opposite to that found in other DNA repair enzymes such as CPD photolyase.⁸ This slow SP repair *in vitro* is unlikely due to the loss of enzyme activity, because our enzyme exhibits a comparable activity with literature findings using either dinucleotide SP TpT^{17,18,23,26,41,42} or a SP TT-containing ss 13-nucleotide as a substrate.⁴⁰ Although these turnover numbers are in sharp contrast to the repair rate of 12.4 min^{-1} reported previously using SP-containing pUC 18 plasmid DNA as a substrate,^{24,25} our studies using pUC 18 plasmid or *E. coli* genomic DNA again found very low SPL activity. The reaction conditions used in these various studies, however, seem not so different. The previous SPL studies employed 0.23 nmole SPL and 17.6 nmole tritium-labeled pUC 18 plasmid containing 435 nmole of SP; the reaction volume was not reported.^{24,25} In our hands, little SPL activity was observed even when 1 nmole of SPL and 57.4 pmole of plasmid (100 μg) containing ~ 6 nmole SP in 400 μL solution were used. The exact reason for this discrepancy is unclear. On the other hand, the activity consistency among most SPL studies conducted to date indicates that SPL activity is likely to be very low with SP-containing oligomers.

Minor DNA conformational changes induced by SP TpT may explain low SPL activity *in vitro*

CPDs and 6-4PPs can both be repaired via the direct reversal mechanism by photolyases.⁸ Photolyases are designed to recognize the dimer or DNA local conformational changes induced by the dimer and subsequently repair the lesion with high efficiency.⁸ To reveal the potential recognition elements by SPL, Singh et al. solved the structure of an SP-containing duplex 16-mer, which reveals that the duplex adopts a right-handed helical conformation with continuous base stacking (Figure 7A).⁵¹ Although the minor groove is widened by $\sim 3 \text{ \AA}$ in the SP-containing strand (Figure 7B), all hydrogen bonding interactions between SP and opposite adenines are maintained, and the overall helical curvature for the SP containing strand still resembles that of a B-form DNA. Given the loss of one hydrogen bond between the 5'-thymine in CPD and its complementary adenine,^{4,53} SP may induce even smaller DNA conformational changes than CPDs. Although the observed minor structural changes were suggested to provide the needed recognition elements for SPL,⁵¹ our enzyme activity studies here indicate that alone these minor changes may not be sufficient to enable effective substrate recognition.

The weak SP TpT recognition by SPL seems to contradict results of a previous DNA digestion study mediated by DNase I.³⁹ This study found the oligonucleotide degradation in a 1 : 1 SP-containing ds-35-mer/SPL complex was about 10-fold slower than in the undamaged ds-35-mer in the presence of SPL, suggesting that SPL may have a higher binding affinity to SP-TpT-containing DNA than the undamaged DNA.³⁹ However, in the same study, the attempt to use a gel-mobility shift assay to examine SP binding affinity to SPL failed, implying a rather weak SP-DNA-protein interaction. Moreover, the DNA footprinting results showed little DNA protection in the 1 : 1 SP-containing ds-35-mer/SPL complex; the suggested DNA bending and protection by SPL in the footprinting assay became obvious only when 5 ~ 10-fold excess of SPL over SP in the oligomer was present.³⁹ Such data then agree with our SPL activity studies reported here in which a relatively high SP repair activity was achieved when a 6-fold ratio of SPL to the SP-containing genomic DNA was used. Therefore, although SPL indeed binds specifically to the SP-containing oligonucleotides, this binding may well be rather weak.

SPL may also flip out the SP lesion from the duplex oligonucleotide before repair occurs,⁴¹ which

explains why dinucleotide SP TpT is an enzyme substrate as it mimics the flipped-out state of SP in the enzyme binding pocket. SPL was suggested to bind to a 9-bp region surrounding SP;³⁹ the phosphate moieties within this region may contribute to enzyme-substrate interaction, that was expected to lead to enhanced enzyme activity,¹⁸ if the SP recognition and flipped-out processes are fast, similar to those observed with CPD/6-4PP photolyases.^{54,55} Such an assumption, however, may not be correct again due to the small SP-induced conformational changes in DNA. Consequently, the repair of dinucleotide SP TpT is faster than in ss-10-mer, ds-20-mer, or plasmid and genomic DNA.

Slow SP TT repair may also be due to small conformational changes The minor DNA conformational changes in SP-containing DNAs may also offer an explanation for the observed slow SP TT repair. The repair of dinucleoside SP TT was found to be 5 - 10-fold slower than dinucleotide SP TpT,^{18,56,57} which is ascribed to the conformational alteration resulting from the loss of the phosphodiester linkage in SP TT, making it fit less well into the SPL binding pocket. SP TT repair was 2~3-fold faster in the ss 13-nucleotide than that in the dinucleoside form, suggesting that the extra bases within the 13-nucleotide assist substrate recognition by SPL. Surprisingly, within a ss oligonucleotide, the repair of SP TpT is much slower than SP TT repair, indicating that the more flexible SP TT structure may be better recognized than the biologically relevant SP TpT, possibly due to weaker stacking interactions among nucleobases as indicated by the lower melting points of a SP TT-containing strand.²⁸ Moreover, repair of SP TT in a ss oligonucleotide leads to strand fragmentation, yielding two shorter oligonucleotides which are expected to exhibit weaker binding affinity toward SPL than the original longer oligomer. The easier product release may well explain the observation that among all substrates tested, only SP TT in ss oligonucleotides can enable more than one SPL turnover upon prolonged enzyme reaction (Table 2). Both factors could be responsible for the faster repair of SP TT than that of SP TpT in a ss oligonucleotide as observed in our studies.

In the duplex environment, the flexibility advantage of SP TT in a ss oligomer seems to no longer exist as reflected by the similar repair rates of SP TT and SP TpT in a ds 20-nucleotide. Such an

observation again can be ascribed to the small DNA conformational changes induced by the lesion. The SP TT structure within a ds 12-nucleotide was solved by Heil (Figure 7C).⁵² Again, all hydrogen bonding interactions between SP and two adenine residues on the complementary strand are maintained. Moreover, the SP TT-containing ds 12-mer's structure can be superposed almost perfectly on the undamaged ds 12-mer structure (Figure 7D), as little structural change was induced by SP TT. Compared with the structure of an oligonucleotide containing a SP TpT lesion shown in Figure 7A, it is likely that both the SP TT- and the SP TpT-containing duplex oligomers possess similar structures with undamaged oligonucleotides. Therefore, although SP TT may be readily recognized and repaired by SPL in a ss oligonucleotide, it is likely difficult for it to be recognized in duplex DNA. As a consequence, its rate of repair by SPL was reduced by 4 - 5 fold in a ds oligomer. The SP TT- and SP TpT-containing 20-mers exhibit similar repair kinetics, further supporting this hypothesis.

Substrate inhibition The similar structures of a SP TpT-containing oligonucleotide and the repaired oligonucleotide shown in Figure 7B indicate that SPL may bind to these two strands with similar affinities and that SPL may have difficulty in replacing the repaired oligonucleotide with another SP TpT-containing oligomer after a turnover. Therefore, SP repair by SPL may not be able to pass the first turnover as shown in all duplex substrates tested. Similar slow product release processes were observed in some DNA glycosylases in which the glycosylase was suggested to bind tightly to the resulting abasic site preventing the enzyme from conducting a second turnover.^{58,59}

Moreover, as shown in SP TpT repair in the ds 20-nucleotide, when the substrate/SPL ratio in the starting solution increased from 1: 1 to 1 : 5, the SPL repair rate was reduced 2 - 3 fold. Such inhibitory behavior was also observed during SP repair in pUC 18 plasmid and *E. coli* genomic DNA. Our SID-LC-MRM-MS analysis revealed that $2 \pm 0.5\%$ and $8 \pm 1\%$ of the thymine residues in the pUC 18 plasmid and *E. coli* genomic DNA, respectively, become SPs. Considering that one fourth of nucleotides are thymidines,⁶⁰ the SPs formed account for $\sim 0.5\%$ and 2% of the nucleotides in the plasmid and genomic DNA used, which is close to the 5% found in ds 20-nucleotide used in our studies. When the amount of

added SPL is close to the number of SPs present, SP repair efficiency is comparable among all SP-containing ds DNA substrates tested. However, the presence of 10-fold more SP in plasmid or genomic DNA almost completely inhibited the enzyme activity, while SPL activity was increased to a level comparable to the repair of the dinucleotide SP TpT with one sixth of the SP substrate present.

The substrate inhibition noted above indicates that undamaged DNAs may also bind to SPL. Moreover, the SPs and undamaged oligonucleotides may prefer to bind to different sites at the enzyme surface. In particular, if different DNAs are competing for the SP binding pocket, even though SPL may favor SP over undamaged nucleotides by a small discrimination factor as indicated by the minor conformational changes observed in duplex DNA, there will always be a certain percentage of enzyme that binds SP no matter how much SP-containing DNA is present. Consequently, only these SP molecules can be repaired and the SPL activity should be proportional to the amount of SP substrates added. However, our studies found higher enzyme activity at lower concentrations of oligonucleotides. We therefore propose that SPL may have some sites remote from its SP binding pocket which also interact with the genomic DNA.

This model is reasonable considering that SPs are the dominant DNA lesion in spores, and have to be repaired during the first few hours of spore outgrowth. Given the minor DNA local conformational changes induced by SP, SPL may have to slide along the genomic DNA to ensure efficient damage recognition across the genome, similar to the lesion recognition mechanism used by DNA glycosylases.⁶¹ To achieve reasonable DNA binding affinity and thus SP repair efficiency, SPL may possess multiple interactions with the genomic DNA besides the SP binding pocket region. As revealed by the SPL crystal structure,⁶² the SPL surface is rich in positively charged regions,⁶² supporting this hypothesis. The continuous genomic DNA structure may ensure that the binding to various sites at the SPL surface is well coordinated. In contrast, when short oligonucleotides are used for SPL studies, they may bind to these sites in a totally random manner. This un-cooperative binding may change SPL to an unfavorable conformation, resulting in a reduced enzyme activity.

SP repair in outgrowing spores may occur with a unique DNA conformation The low SPL activity observed *in vitro* may not reflect that in outgrowing spores because in order to carry out the majority of SP repair, an activity of several turnovers per minute is likely needed.^{63,64} As shown by the *E. coli* UvrABC endonuclease that is involved in NER, the UvrA protein recognizes the DNA damage, and UvrB bends the DNA before the UvrB-UvrC complex catalyzes DNA backbone cleavage.^{65,66} Similarly, SPL may have to team with other protein(s) for damage recognition and repair *in vivo*. These proteins would be missing in our *in vitro* studies, which may result in the low SPL activity observed.

Alternatively, repair of SP in outgrowing spores may not occur in genomic DNA adopting the general conformation found in a normal vegetative cell, which typically possesses a B-conformation. The dormant spore genome is saturated by a group of DNA-binding proteins termed small, acid-soluble spore proteins (SASPs),^{4,67-69} which account for ~ 10% of total spore core protein depending on the species analyzed,⁷⁰ and these cause the DNA to adopt a tightly packed toroidal morphology in an A-like conformation.^{71,72} SASPs continue to bind to genomic DNA for an extended period of time in germinated and outgrowing spores before they dissociate from DNA,^{73,74} thus helping maintain the genomic DNA in an A-like conformation.⁷⁵⁻⁷⁸ During spore germination and then outgrowth, it likely takes time for this large amount of SASPs to completely dissociate from DNA. However, >70% of SPs are repaired during the first hour of germination and outgrowth.⁴⁶ It is thus possible that SPL-mediated SP repair may take place within the tightly packed DNA toroids or synergistically with SASP dissociation when DNA still adopts an A-like conformation.

The hypothesis noted above is supported by studies of double-strand break (DSB) repair mediated by the non-homologous-end joining (NHEJ) pathway in outgrowing spores. DSB formation is a major DNA damage event in spores under UVA or full spectrum sunlight,^{79,80} and repair of DSBs by the NHEJ pathway plays an important role in spore survival.^{81,82} It has been suggested that the tightly packed DNA toroids provide the framework to enable NHEJ repair in outgrowing spores.⁸³ In addition, some unique low molecular weight ATP-dependent ligases may facilitate access of the NHEJ enzymes to DSBs within

the tightly packed toroids.⁸³

SP repair by SPL in outgrowing spores was found to require energy,⁶³ making SPL different from photolyases which use light for catalysis but do not consume ATP. Given that SP repair by SPL in outgrowing spores occurs around the same period as DSB repair by NHEJ, SPL may also have to function within the DNA toroid structure or shortly after the toroid collapses into the more compacted nucleoid in outgrowth,^{78,84,85} where DNA still largely adopts an A-conformation. Such a repair process in the tightly packed toroid architecture may need ATP to unfold the complex, making the SPs accessible to SPL. If this is true, the lack of the “correct” DNA conformation resulting from ATP-associated DNA unfolding may readily explain the relatively low SPL activity exhibited on SP-containing *E. coli* genomic DNA or duplex 20-mer.

SAM regeneration The slow SP repair supported by DNA adopting a general B-like conformation may also explain the lack of SAM regeneration in most of the *in vitro* SPL studies so far. As shown in Figure 2, the conserved cysteine is the H-donor; a thiol radical will be generated on this residue, which likely oxidizes the neighboring tyrosine (Y99 in *Bs* SPL), converting it to a tyrosyl radical. These protein radicals have to be reduced before the next turnover. The needed electron may come from protein-harbored redox cofactors; however, such a possibility is unlikely because no other factors besides the [4Fe-4S] cluster involved in SAM cleavage is revealed by the recently solved SPL crystal structure.^{23,62} Therefore, the abstraction of an H-atom from 5'-dA and subsequent SAM regeneration as proposed in the current mechanism is the most reasonable hypothesis to complete a catalytic cycle. However, for a long time, SAM regeneration was not convincingly observed during SP repair *in vitro*. One study showed that one molecule of SAM supports > 500 turnovers using SP-containing pUC 18 plasmid DNA.²⁴ However, this conclusion was not well supported by our pUC 18 plasmid studies here, nor by other studies using dinucleotide SP TpT,^{18,26,41} dinucleoside SP TT⁵⁶ or SP TT-containing oligonucleotides⁴⁰ as enzyme substrates.

Although not reaching > 500 turnovers, our results using pUC 18 plasmid and *E. coli* genomic DNA

did provide strong evidence for SAM regeneration by observing a ratio of ~ 8 between SP repaired and 5'-dA generated under single-turnover conditions. Our results imply that a long oligonucleotide is needed to enable such a process in SPL catalysis. The different SAM regeneration pattern between SP-containing *E. coli* DNA and SP-containing short oligonucleotides again indicates that SPL may have remote DNA binding sites, which are bound in a cooperative manner with the SP binding site when a long genomic DNA is used as an enzyme substrate. The binding of DNA at this other site may subsequently change SPL protein conformation, enabling SAM regeneration at the end of the catalytic cycle.

SPL is a rare example among DNA repair enzymes In summary, by surveying a number of SP substrates embedded within an oligonucleotide as SPL substrates, extremely low SP repair efficiency was observed. These observations make SPL a special case among major DNA repair enzymes, where an enzyme typically exhibits a high affinity toward its substrate lesion to ensure effective lesion removal in the genome. The low SPL activity is in sharp contrast to DNA photolyases, which, like SPL, also use the direct reversal strategy to repair CPDs and 6-4PPs. Photolyases repair pyrimidine dimers in the ss or ds context with high efficiency; they also repair the dimers in dinucleotide context but with much reduced efficiency.⁸ SPL exhibits a totally opposite trend, showing the highest efficacy with dinucleotide SP TpT. Its efficiency toward SP in an oligonucleotide or genomic DNA is so low that it cannot reflect rates of SP repair in outgrowing spores. As genome stability is essential for an organism's survival, any DNA repair enzyme that cannot efficiently fulfill this designated function will not survive during millions of years of evolution. Considering that SPL is the primary DNA repair enzyme in germinating spores, it is almost certain that spores not only possess a special mechanism to alter the DNA conformation making SP formation the dominant DNA UV photochemistry,^{1,4,68,69} but also possess a unique mechanism/cellular environment to facilitate efficient SP repair, which might be very slow in growing cells. Therefore, although both DNA photolyases and SPL use direct reversal mechanism for DNA repair and likely share a common enzyme ancestor,¹⁶ they appear to work under very different environments *in vivo*.

It is now known that in dry or desiccated microorganisms exposed to UVC or UVB photons, SP is

formed as a major DNA photo-lesion.⁸⁶ SP was also formed in UV irradiated dried yeast cells,^{87,88} and the yeast cells likely carrying SP exhibit greatly increased UV sensitivity. SP formation has been suggested to occur in UV-irradiated frozen bacteria, bacteriophage,⁸⁹ and yeast,⁹⁰ and the frozen cells appear to be more sensitive to UV irradiation possibly again due to the presence of SP. In these non-spore forming organisms, SP repair is likely to be conducted by other DNA repair pathways such as NER.⁹¹ If the designated SP repair enzyme in spores, SPL, has problems in recognizing and repairing SP in DNA with a normal B-conformation, it is likely that SP may also not be efficiently repaired by NER in these non-spore forming species, and left unrepaired SP may induce mutations during a translesion synthesis event. Indeed, a study using competent cells of *Haemophilus influenzae* found that the presence of SP increases the mutation rate by several hundred fold compared with undamaged control DNA,⁹² suggesting that the strong mutagenic tendency from unrepaired SPs may be responsible for SP toxicity in vegetative cells. Although these questions are not directly related to the SPL mechanistic studies discussed in this report, they are somewhat related by the indicated biology and will need to be addressed in the future.

Author Contributions L.Y. and Y.J. conducted the enzymology studies and oligonucleotide syntheses respectively. L.L and P.S. wrote the manuscript.

Notes The authors declare no competing financial interest.

ACKNOWLEDGMENT This work was supported in part by National Institutes of Health Grant ES017177 and the National Science Foundation CHE1506598 (to L.L.). The NMR and MS facilities at IUPUI are supported by National Science Foundation MRI grants CHE-0619254 and DBI-0821661, respectively. We thank Professor Yinsheng Wang at University of California Riverside for helpful discussions on SP quantification via mass spectrometry.

Supplementary Data: Characterizations of the SP-containing 13-mer oligonucleotide, HPLC chromatograms of SPL reactions.

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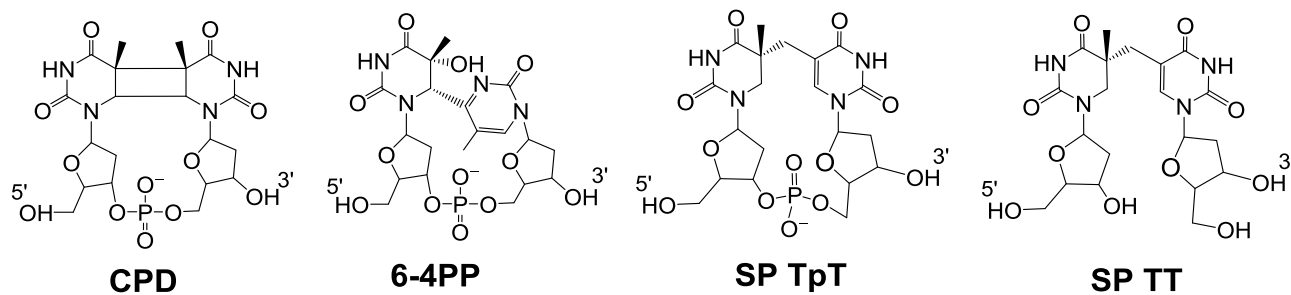


Figure 1. Chemical structures of thymine dimers in the dinucleotide or dinucleoside context. All these dimers can be repaired via direct reversal mechanisms when present in the genomic DNA. CPDs and 6-4PPs are repaired by specific light-dependent photolyases and SP is repaired by SPL which is light-independent but energy-dependent.

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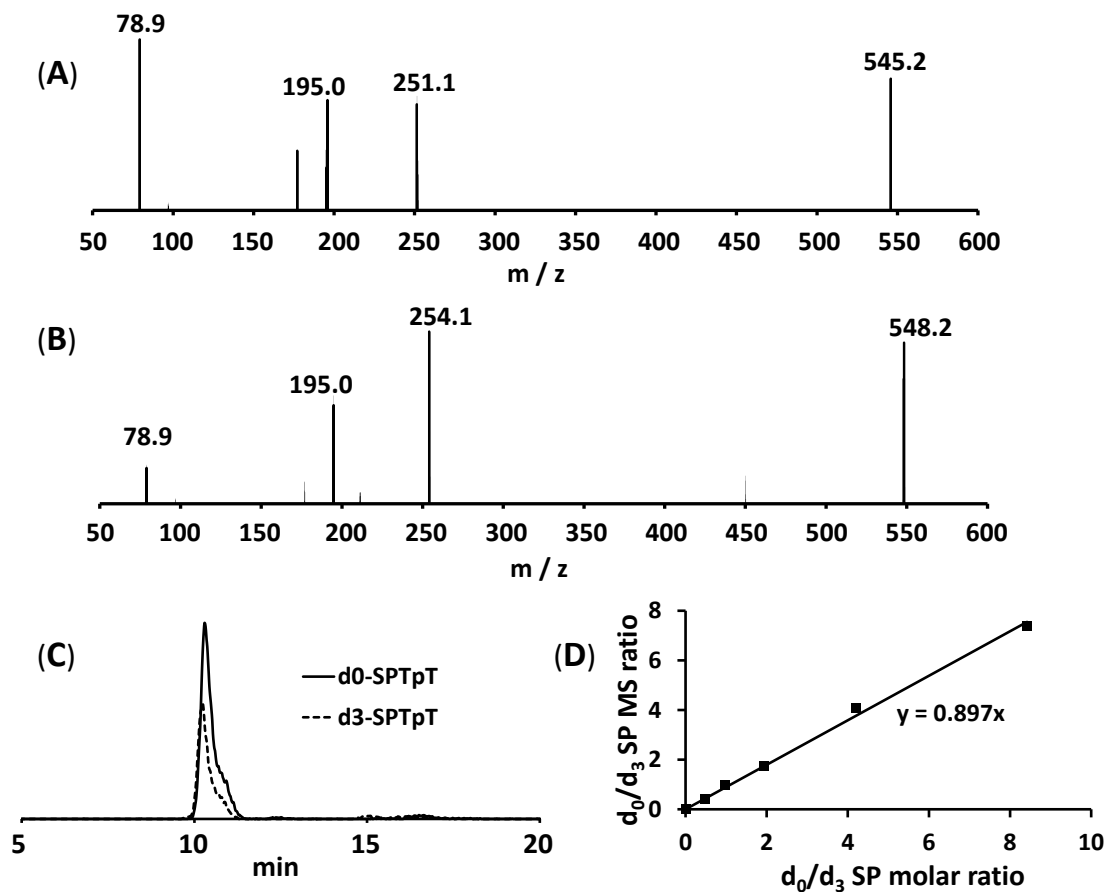


Figure 3. (A) ESI-MS spectrum of d_0 -SP TpT monitored under the negative ion mode. The parent ion (m/z 545.2) and major fragmentation signals with m/z of 251.1, 195.0, and 78.9 were observed. (B) ESI-MS spectrum of d_3 -SP TpT monitored under the negative ion mode. The parent ion (m/z 548.2) and major fragmentation signals with m/z of 254.1, 195.0, and 78.9 were observed. (C) A typical ion extraction chromatogram when a known amount of d_3 -SP TpT was used as the internal standard to quantify the amount of d_0 -SP TpT formed in UV irradiated pUC 18 plasmid or *E. coli* genomic DNA. (D) The calibration curve between mass signal integration ratio and mole ratio generated using known amounts of d_0 -SP and d_3 -SP. The d_0 -SP and d_3 -SP exhibit slightly different ionization behavior in the mass spectrometer as reflected by the slope of 0.9 in the calibration curve.

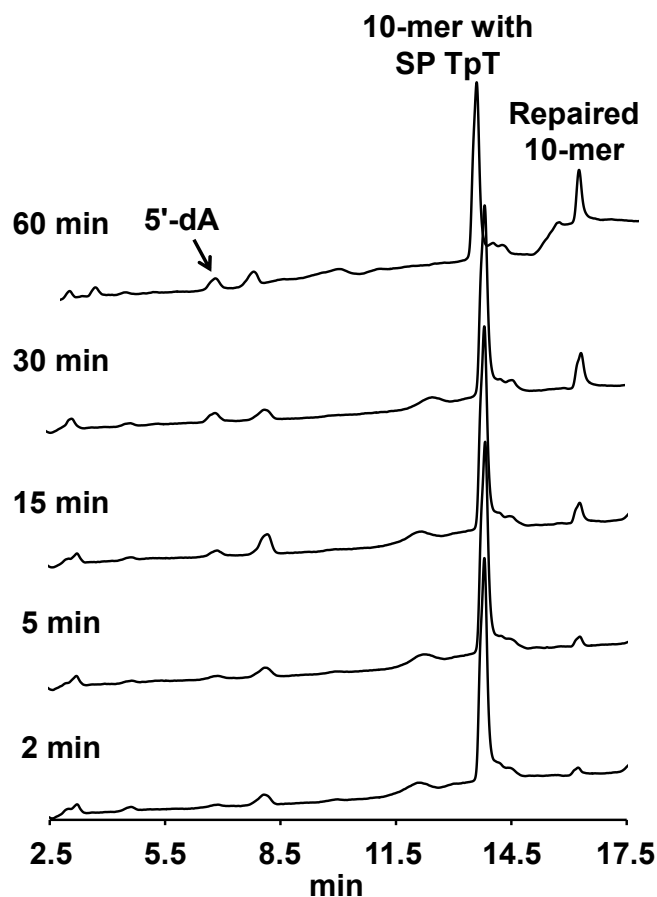


Figure 4. HPLC chromatograms of SP TpT repair in the ss 10-mer d(CACC[SP TpT]CATC) mediated by SPL. The starting reaction solution contained 1 nmole SPL, 5 nmole SAM, and 2 nmole oligonucleotide in 200 μ l pH 7.0 Tris-HCl buffer containing 25 mM Tris, 250 mM NaCl and 10% glycerol. At 60 min, only 0.4 turnovers were obtained. The reaction also generated 5'-dA, which is about two thirds of the repaired SP, suggesting the reaction to be only partially catalytic.

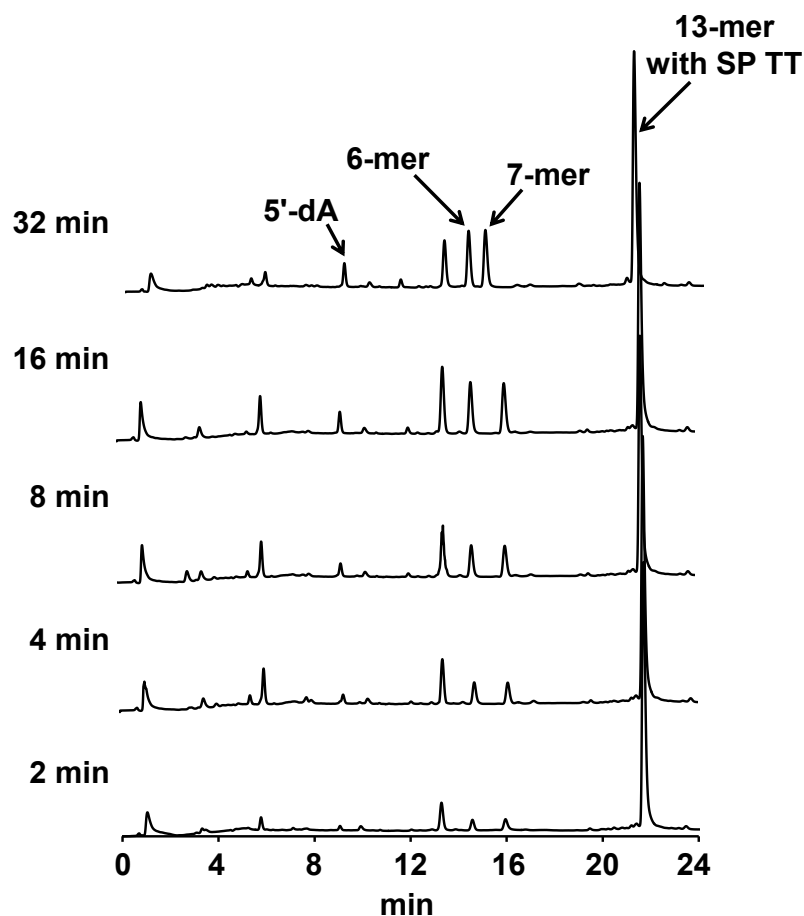


Figure 5. HPLC chromatograms of SPL-mediated SP TT repair with the ss 13-mer d(CGTGA[SP TT] ACAGCC). The starting reaction contained 1 nmole SPL, 5 nmole SAM and 3 nmole 13-mer oligonucleotide in 200 μ l 25 mM Tris-HCl buffer containing 250 mM NaCl and 10% glycerol at pH 7.0. Repair of SP TT results in breakage of the 13-mer into a 6-mer d(CGTGAT) and a 7-mer d(TACAGCC). The reaction slowed after 5 min and an overnight reaction resulted in \sim 1.2 turnovers.

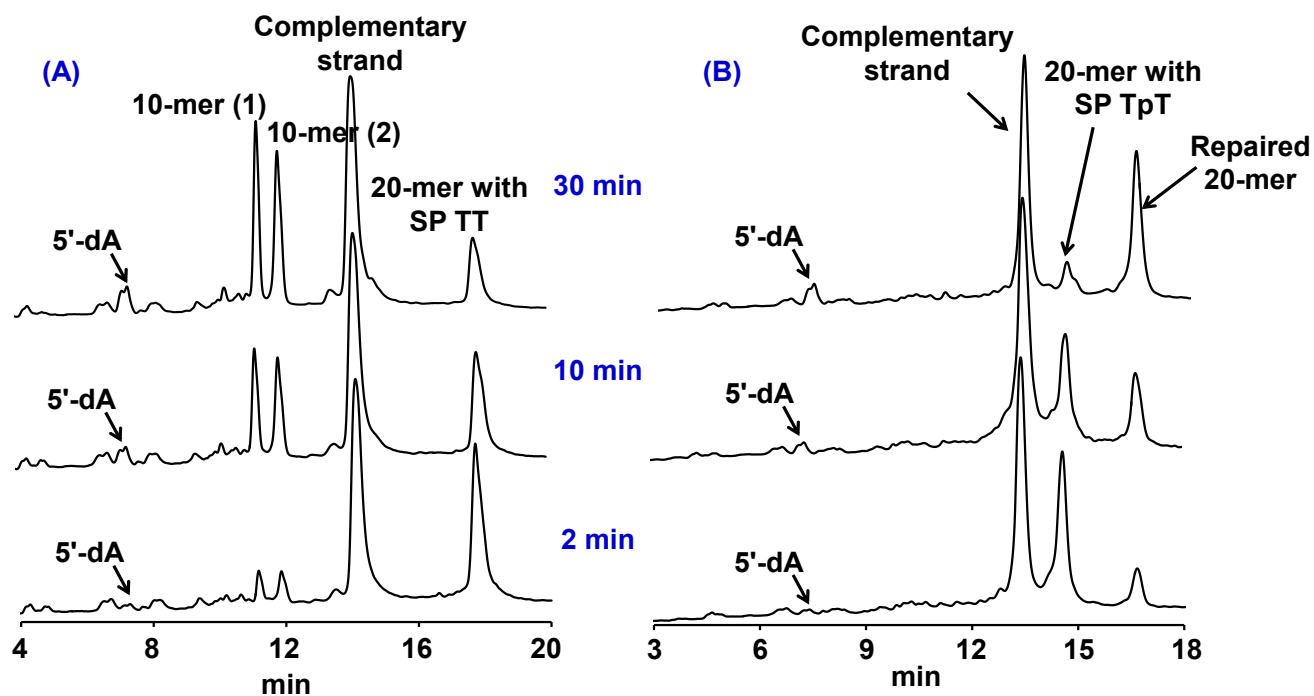


Figure 6. (A) HPLC chromatograms of SP TT repair in the ds 20-mer d(CTCGACACG[SP] CGCATGCCA)/d(TGGCATGCGAACGTGTCGAG) mediated by SPL. SP TT repair results in breakage of the 20-mer strand into two 10-mers, which can be separated by HPLC operated at 60 °C and integrated to allow determination of the repair rate. (B) HPLC chromatograms of SP TpT repair in the ds 20-nucleotide with the same sequence. Both reaction solutions contained 1 nmole SPL, 5 nmole SAM and 1 nmole 20-mer in 200 μ l 25 mM Tris-HCl buffer containing 250 mM NaCl and 10% glycerol at pH 7.0. SPs were repaired in both strands with similar rates of $0.03 \pm 0.01 \text{ min}^{-1}$.

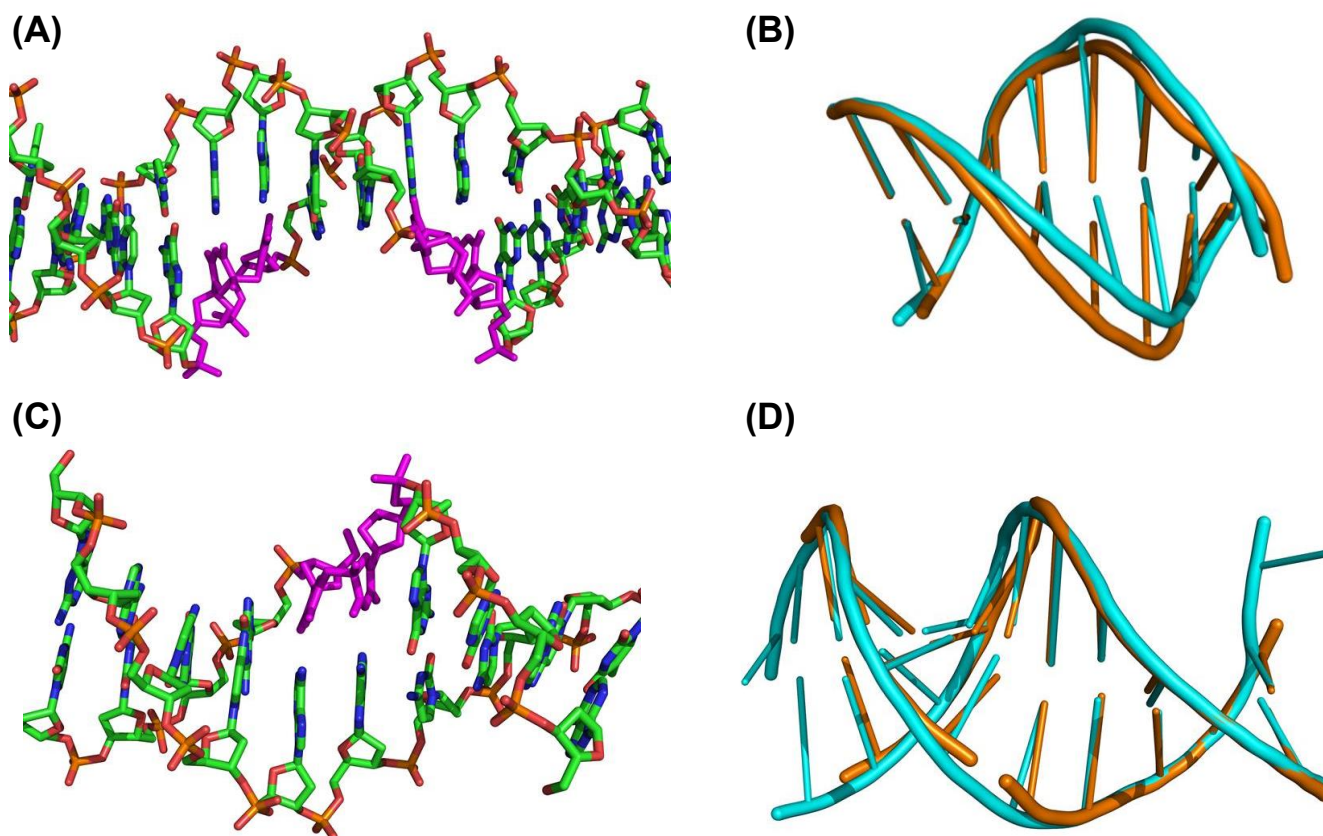


Figure 7. (A) Crystal structure of the 16 bp duplex with two SP dimers (shown in magenta) crystallized in a nucleoprotein complex which also contains two molecules of the N-terminal fragment of *Moloney murine leukemia virus reverse transcriptase* (MMLV RT) (pdb entry 4M94).⁵¹ The asymmetric unit of the crystal includes one protein molecule and eight bps of the duplex oligomer. The right-handed double helical structure and all H-bonding interactions in the duplex are maintained. (B) Superimposed structures of the 8-bp asymmetric unit of the undamaged oligomer (in cyan, pdb entry 4M95) and the SP TpT-containing oligomer (in orange) bound to MMLV RT. The SP-containing strand exhibits a widened minor groove. Such a structural change, however, is considered minor and may not support efficient lesion recognition by SPL. (C) Crystal structure of the SP TT-containing 12-bp duplex in a nucleoprotein complex formed between *Geobacillus stearothermophilus* DNA polymerase I and the 12-bp oligonucleotide strand (pdb entry 2YLJ).⁵² The right-handed double helix and all H-bonding interactions in the 12-bp sequence are maintained. (D) Superimposed structures of the undamaged oligomer (in cyan, pdb entry 1NJY) and the SP TT-containing oligomer (in orange). The two structures overlay nearly perfectly except for the broken backbone in SP TT.

Table 1. Oligonucleotides synthesized for SPL activity studies	
Oligonucleotide name	Oligonucleotide sequence
SP TpT-containing 10-mer	d(CACC [SP TpT] CATC)
SP TT-containing 13-mer	d(CGTGA [SP TT] ACAGCC)
SP TT-containing 20-mer	d(CTCGACACG [SP TT] CGCATGCCA)
SP TpT-containing 20-mer	d(CTCGACACG [SP TpT] CGCATGCCA)

Table 2. SPL activity reflected by the initial reaction rate (min^{-1}) and overall turnover numbers using SP TT- and SP TpT-containing oligonucleotides.

Oligonucleotides used	Mole ratio SPL / SP	SP repair rate (min^{-1})	Ratio of SP repaired / 5'-dA generated	Turnovers after an overnight reaction
ss SP TpT- containing 10-mer ^a	1 : 2	0.007 ± 0.002	1.4 ± 0.2	n. d.
ss SP TT- containing 13-mer ^a	1 : 3	0.08 ± 0.02	1.1 ± 0.2	1.1 ± 0.2
ds SP TT- containing 13-mer ^a	1 : 2	0.04 ± 0.02	0.9 ± 0.2	1.0 ± 0.2
ss SP TT- containing 20-mer ^a	1 : 2	0.09 ± 0.02	1.3 ± 0.2	n. d.
	1 : 5	0.03 ± 0.01	1.1 ± 0.2	1.5 ± 0.3
ds SP TT- containing 20-mer ^a	1 : 1	0.03 ± 0.01	1.1 ± 0.2	0.6 ± 0.2
	1 : 5	0.01 ± 0.002	0.5 ± 0.1	0.7 ± 0.2
ds SP TpT- containing 20-mer ^a	1 : 1	0.03 ± 0.01	1.2 ± 0.2	0.5 ± 0.2
	1 : 5	0.01 ± 0.002	0.4 ± 0.1	0.7 ± 0.2
Dinucleotide SP TpT ^b	1 : 20	0.32 ± 0.05	1.5 ± 0.2	7.5 ± 2.5
SP TpT-containing pUC 18 plasmid ^c	1 : 1	0.05 ± 0.03	3 ± 1	0.3 ± 0.1
	1 : 10	< 0.001	n. d.	n. d.
SP TpT-containing <i>E.</i> <i>coli</i> genomic DNA ^c	6 : 1	0.22 ± 0.03	8 ± 1	0.05 ± 0.01
	1 : 1	0.06 ± 0.02	3 ± 1	0.3 ± 0.1
	1 : 10	< 0.001	n. d.	n. d.

a. Determined by HPLC assay.

b. Determined by both HPLC and SID-LC-MRM-MS assays.

c. Determined by SID-LC-MRM-MS assay.