Experimental Section:

General:

The *Escherichia coli* strain Tuner(DE3)pLysS (Novagen) and the pDEST17 expression vector (Invitrogen) were used for overexpression of SplG. The BugBuster 10X protein-extraction reagent was purchased from Novagen and the Ni Sepharose 6 FastFlow was from GE Healthcare. *S*-Adenosylmethionine was obtained from Sigma-Aldrich and used without further purification. Oligonucleotide primers and counter strands for dsDNA repair reactions were obtained from Metabion. Other chemicals, solvents and molecular biology reagents were purchased from Invitrogen, Thermo Fisher Scientific, Fermentas, NewEnglandBiolabs, Merck, Sigma-Aldrich, Alfa Aesar, Acros or Fluka in the highest qualities.

Substrate synthesis:

Oligonucleotides containing the spore photoproduct and the spore photoproduct dinucleoside were synthesized using published procedures.[1] Fluorescent oligonucleotides were synthesized on a 394 DNA/RNA Synthesizer (Applied Biosystems) using DyLight DY547 (Glen Research).

Cloning of SPL mutant expression vectors:

The gene coding for the SplG (UniProt accession ADU94823) from *Geobacillus stearothermophilus* DSM No. 22 was cloned into the pDEST17 expression vector coding for an N-terminal His_6 tag as previously described.[1] Site-directed mutagenesis was performed using the Phusion™ site-directed mutagenesis kit from Finnzymes. Following DNA primers were used: 5′-Pho-GCCACGTCCGACATTGTCGGGATCG-3′ and 5′-Pho-TGCCGCCTCGAAGCGAGTGATC-3′ for C140A, 5′-Pho-GGCACGTCCGACATTGTCGGGATC-3′ and 5′-Pho-TGCCGCCTCGAAGCGAGTGATC-3′ for C140G, 5′-Pho-TTTTCGAAACGACATTAGGAAGCAAGCC-3′ and 5′-Pho-AGCAATAATGGCAATGGCCCATGC-3′ for Y98F. Standard sequencing of cloned plasmid constructs was performed by GATC Biotech.

Expression and purification of recombinant SP lyase:

For protein expression, transformed Tuner(DE3)pLysS (Novagen) cells were grown in Luria Bertani medium supplemented with carbenicillin (100 μg mL^{-1}) and $FeCl_3$ (100 μM) at 37 °C. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 22 °C (OD_{600} = 0.7-0.9) and incubated for 2 h. Cells were harvested by centrifugation and transferred into a glovebox and all following steps were performed under anaerobic conditions. Cell lysis was carried out using 10X BugBuster (Novagen) in 50 mM HEPES, 100 mM NaCl, 10 mM imidazole and 5 % glycerol, pH 7.5. The protein was purified by nickel-affinity chromatography in a batch method. The column was washed with 50 mM HEPES, 100 mM NaCl, 20 mM imidazole, 0.5 % Tween-20, 5 % glycerol, and 5 mM β-mercaptoethanol, pH 7.5, and protein was eluted with 50 mM HEPES, 100 mM NaCl, 250 mM imidazole, and 5 % glycerol, pH 7.5. The protein extract was then further purified by heparin-affinity chromatography in a batch method. The column was washed with 50 mM HEPES, 100 mM NaCl, and 5 % glycerol, pH 7.5 and SplG was eluted in >90 % purity with

50 mM HEPES, 300 mM NaCl, and 5 % glycerol, pH 7.5. The protein sample was concentrated by centrifugal filter devices and stored at -20 °C.

DNA repair assays:

Enzyme assays were usually performed with 1 µM SplG supplemented with 1 mM SAM and substrate in 50 mM HEPES, 50 mM NaCl, and 5 % glycerol, pH 7.5 under anaerobic conditions. The reaction was started with the addition of 1 mM dithionite and incubated at 35 °C. Flash-freezing with liquid nitrogen and subsequent exposure to air was used to stop the reaction. For time-dependent measurements aliquots with appropriate substrate concentration for HPLC analysis (250 pmol for UV detection and 2 to 30 pmol for fluorescence detection) were taken out at various time points. The endpoint fraction was taken after overnight incubation. For determination of K_M value the time frame of the linear initial velocity was firstly measured by a time dependent study at 0.1 µM substrate concentration. The substrate dependent study was then conducted at 45 min for *wt* SplG and all mutants. Fluorescently labeled ODN 1 was used as substrate for higher sensitivity for K_M measurements. For other repair experiments non-labeled ODN 4 was used. Assays were performed with dsDNA using ODN 2 and ODN 5 as counter strands, respectively.

Protein, iron, and sulfide assays:

The protein concentration was measured using the Bradford method.[2] Iron and sulfide content were determined as described by Fluhe et al[3] using the methods of Beinert[4] and Kennedy.[5] The Fe^{2+}-ferene complex and sulfide-DMPD samples were analyzed with a Jasco V-650 spectrophotometer. Iron and sulfur contents were determined to be 1.7 ± 0.4 Fe^{2+} atoms per protein and 1.5 ± 0.0 S^{2-} atoms per protein. In the assumption that mainly $[Fe_4S_4]$ clusters are present, SplG fractions are loaded to 37.5 % with an active cluster. Protein concentrations were corrected by this factor to give the active protein concentration needed for the calculation of the correct substrate turnover rates.

HPLC:

The 0.2 µm-filtered reaction mixtures were loaded onto HPLC for detection of ODNs and 5´-dAdoH. A Waters system (Alliance 2695 with PDA 2996 and 2475 Multi-Wavelength Fluorescence Detector) with EC 250/3 Nucleodur C18, Gravity, 3 µm column from Macherey-Nagel was used. Residual protein was removed by a CC 8/3 Nucleodur C18 Gravity, 3 µm prefilter. Separation took place with a gradient of 0.1 M triethylamine/acetic acid in water and 80 % acetonitrile. For denaturing of dsDNA, HPLC was performed at 60 °C.

HPLC data analysis:

Oligonucleotide and 5´-dAdoH signals were identified by their retention time and their specific PDA signal, integrated and corrected with their extinction coefficient at 260 nm. SP repair was calculated out of the mean area of products divided by starting material plus mean area of products. 5´-dAdoH concentration was determined in relation to product concentration. Detection of starting material and product strands was simplified using the fluorescent dye DyLight DY547. No correction of integrated areas was necessary. Data were

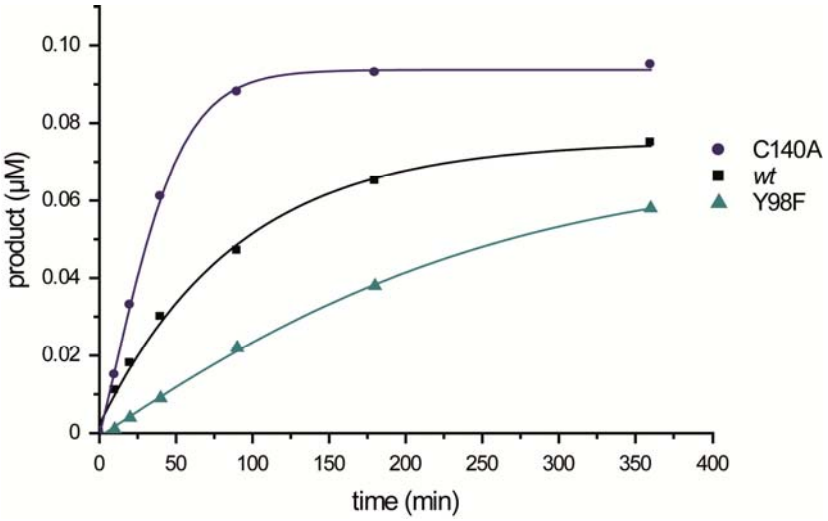
analyzed with Origin 6.0. K_M value and V_{max} estimations were performed with SigmaPlot 11.2.

UV-VIS:

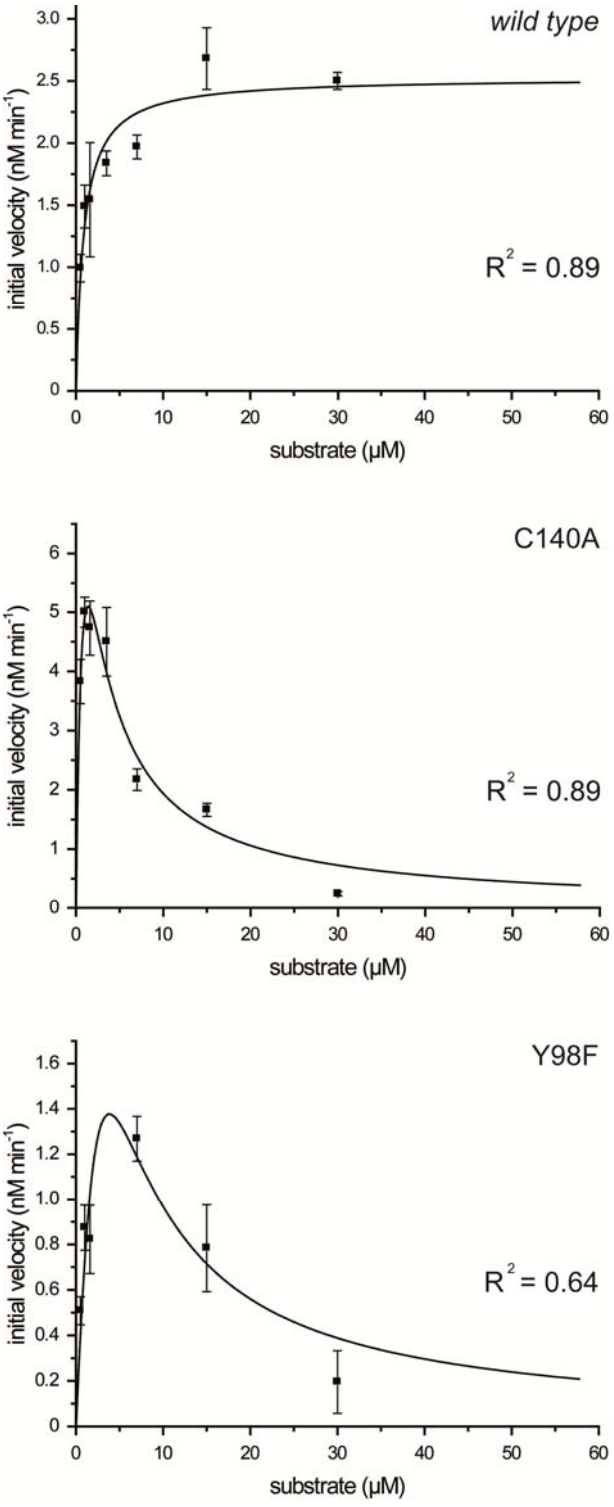
UV-VIS spectra from 300 to 600 nm were taken in a 1 cm cuvette plugged for anaerobic conditions using a Jasco V-650 spectrophotometer. Two sets of studies were conducted after baseline correction measurement with a buffer sample. In the first set, 80 µM SplG *wt* or Y98F were analyzed either alone or after 3 h reduction with 3 mM dithionite. Secondly, 80 µM SPL supplemented with 150 µM SAM and 460 µM spore photoproduct dinucleoside were analyzed prior to and after 3 h incubation with 3 mM dithionite. Both reaction sets were performed at 35 °C in 50 mM HEPES, 210 mM NaCl, and 5 % glycerol, pH 7.5. Repair of substrate was checked with HPLC analysis. SP dinucleoside at a low saturating concentration was chosen to slow down the reaction significantly so that protein has to endure in its resting state for a longer time period. Since a characteristic tyrosyl radical signal overlaps with an [4Fe4S] cluster signal at approximately 410 nm, the two spectra taken during *wt* and Y98F repair reaction were subtracted from each other. Assuming that due to the huge excess of dithionite during the repair reaction the [4Fe4S] cluster stays equally reduced in both proteins, the subtraction should show differences caused by Y98.

Table S1. Depiction of the prepared oligonucleotides:

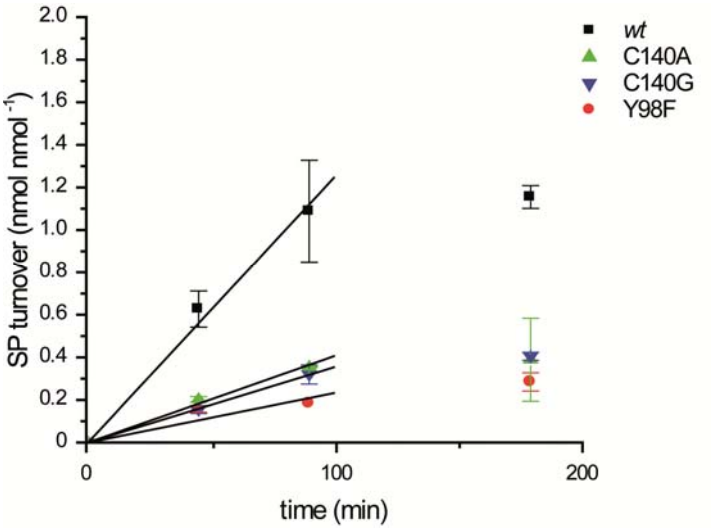
Number	**Sequence (5′ → 3′)**
ODN 1	Dy547-ACAGCGG (SP) GCAGCT
ODN 2	ACCTGCAACGCGTGT
ODN 3	Dy547-ACAGCGGGT
ODN 4	CAGCGG (SP) GCAGG
ODN 5	CCTGCAACCGCTG
ODN 6	CAGCGGT
ODN 7	TGCAGG



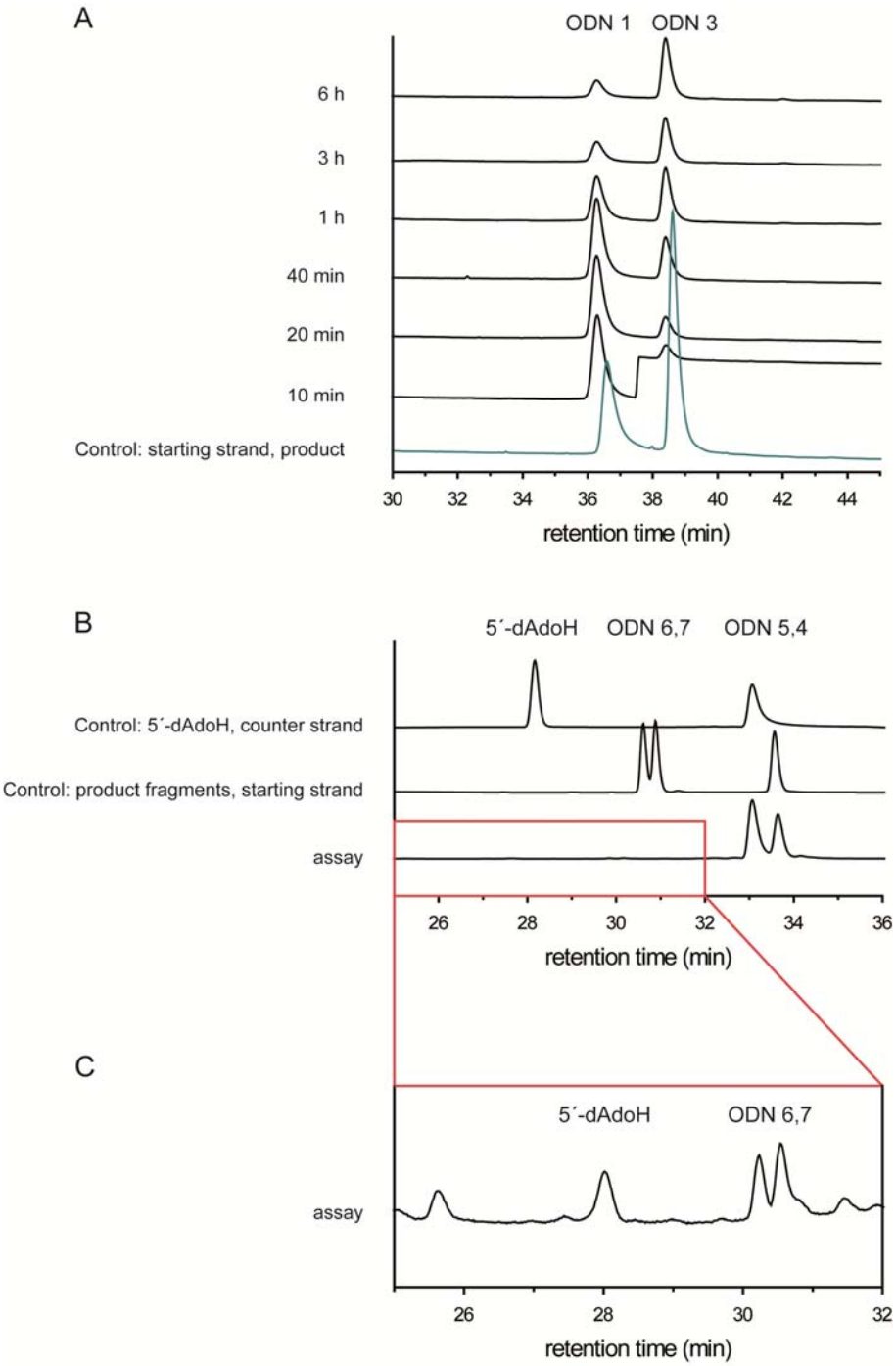
Supplementary figure S1. Time curves of SplG *wt* and mutant proteins at 0.1 µM substrate concentration. The formation of ODN 3 was linear with time up to 50 min for all three proteins. The K_M value was subsequently determined at 45 min to guarantee initial velocity.



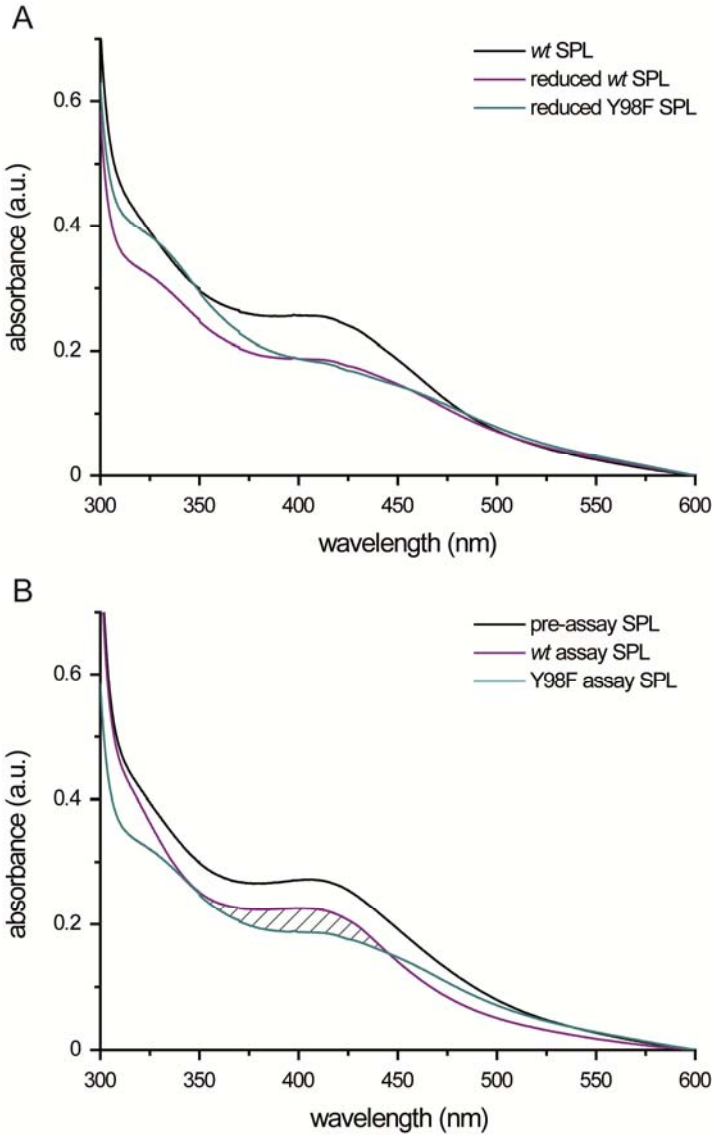
Supplementary figure S2. Substrate dependent repair assays of SplG *wt* and mutant proteins. For the repair reaction ODN 1 hybridized together with ODN 2 was used as dsDNA substrate. The K_M value and V_{max} were estimated using SigmaPlot Enzyme Kinetics Software 11.2.



Supplementary figure S3. Time dependent repair reactions of SplG *wt* and mutant proteins with a substrate concentration of 10fold *wt*K_M. The first data points were used for linear fitting to calculate an approximation of V_{max} for comparison of *wt* and mutant proteins. The slopes resulting from this are 0.012 ± 0.0005 min^{-1} for *wt* SplG, 0.0041 ± 0.0002 min^{-1} for C140A, 0.0035 ± 0.0000 min^{-1} for C140G and 0.0023 ± 0.0004 min^{-1} for Y98F.



Supplementary figure S4. Representative HPLC chromatograms of repair reactions with fluorescently labeled DNA substrate (**A**) and with non labeled DNA substrate (**B** and zoom in **C**).



Supplementary figure S5. A SPL *wt* as isolated (black) and SPL *wt* (purple) and Y98F (cyan) reduced with 3 mM dithionite for 3 h. **B** SPL *wt* in pre-assay conditions with SP dinucleoside substrate and SAM added (black) and SPL *wt* (purple) and Y98F (cyan) 3 h after addition of 3 mM dithionite. The diagonally shaded area depicts differences between *wt* and Y98F assay spectra. The subtraction of both spectra is shown in the main text. HPLC assay analysis showed that *wt* protein repaired 4 µM and Y98F 3 µM SP dinucleoside during the reaction. Hence, 5 % and 4 % of protein, respectively, were in their resting state, which possibly includes a radical at Y98.

Supplementary figure S6. Sequence analysis of spore photoproduct lyase enzymes from different bacteria classes. Only the section from E81 to F160 of SplG from *Geobacillus stearothermophilus* is shown. The critical Y98 residue is highlighted in purple. Conserved residues are shown in red, non-conserved in light blue.

References:

1. K. Heil, A. C. Kneuttinger, S. Schneider, U. Lischke and T. Carell, *Chem. Eur. J.*, 2011, **17**, 9651-9657.
2. M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248-254.
3. L. Fluhe, T. A. Knappe, M. J. Gattner, A. Schafer, O. Burghaus, U. Linne and M. A. Marahiel, *Nat. Chem. Biol.*, 2012, **8**, 350-357.
4. H. Beinert, *Anal. Biochem.*, 1983, **131**, 373-378.
5. M. C. Kennedy, T. A. Kent, M. Emptage, H. Merkle, H. Beinert and E. Munck, *J. Biol. Chem.*, 1984, **259**, 14463-14471.