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Spore Photoproduct Lyase Catalyzes Specific Repair of the 5*R* but not the 5*S* Spore Photoproduct

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Bacterial spores are remarkable in their resistance to chemical and physical stresses, including exposure to UV radiation. The unusual UV resistance of bacterial spores is a result of the unique photochemistry of spore DNA coupled with the efficient repair of accumulated damage. Exposure of bacterial spores to UV radiation results in the formation of a methylene-bridged thymine dimer, 5-thyminyl-5,6-dihydrothymine (spore photoproduct, or SP), as the primary photoproduct.[1–3] SP accumulates in UV-irradiated spores, however it is rapidly repaired upon germination, thus giving rise to the extraordinary UV resistance of bacterial spores.[4,5] The repair of SP is catalyzed by the enzyme spore photoproduct lyase (SPL), and involves the direct reversal of SP to two thymines without base excision (Scheme 1).

SPL is a member of the radical AdoMet superfamily, and utilizes a [4Fe-4S] cluster and S-adenosylmethionine (AdoMet) as essential cofactors in SP repair.[6–9] We have previously shown that SP repair is initiated by abstraction of H• from C6 of SP by an AdoMet-derived 5′-deoxyadenosyl radical;[10,11] this H-atom abstraction is thought to initiate a radical-mediated β-scission of the C5-C_{bridge} bond in the photoproduct, as originally proposed by Mehl and Begley.[12]

While two distinct diastereomers of SP (5*R* or 5*S*, Fig 1) could in principle be formed upon UV irradiation of bacterial spores, only the 5*R* configuration is possible for SP formed from adjacent thymines in double helical DNA, due to the constraints imposed by the DNA structure.[13] The 5*S* configuration, therefore, is possible only in less well-defined DNA structures or as an interstrand crosslink. It was thus quite surprising when two recent reports concluded that SPL repairs only the 5*S*, and not the 5*R*, isomer of a synthetic SP substrate.[14,15] We report here results from HPLC and MS analysis of *in vitro* enzymatic assays on stereochemically-defined synthetic SP substrates demonstrating that SPL specifically repairs only the 5*R* isomer of SP. This stereospecific repair of 5*R*-SP by SPL is consistent with the longstanding hypothesis that SP is a result of UV-induced dimerization of adjacent thymines in double-helical DNA.

SPL was cloned from *Clostridium acetobutylicum*, overexpressed in *Escherichia coli*, and purified using a method similar to published procedures.[11] The enzyme contained 2.9 (± 0.2) Fe per SPL, and had UV-visible and EPR spectroscopic properties characteristic of an iron-sulfur enzyme. The 5*R* and 5*S* diastereomers of protected (N-SEM, O-TES, and O-TBDMS) SP were synthesized using modifications of published procedures,[13,15] and were subsequently deprotected (Supporting Information). The structures of the fully protected, the di-SEM protected, and fully deprotected dinucleoside spore products were confirmed by ^{1}H and ^{13}C NMR techniques, and NOESY and ROESY were used to assign the stereochemistry at C-5 (S.I.). In order to remove any potential ambiguity associated with the assignment of stereochemistry at C-5 in the open dinucleoside forms of SP, the protected open dinucleosides

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(5*R* and 5*S*) were converted to closed cyclic phosphotriesters, and the stereochemical assignments at C-5 were confirmed by NOESY and ROESY (S.I.). Stereochemical assignments were based on initially assigning the pro-*S* and pro-*R* hydrogens at C6, with the pro-*R* hydrogen appearing downfield of the pro-*S* hydrogen for nearly all of the synthetic forms of both diastereomers of SP (S.I.). Our assignment of pro-*R* and pro-*S* hydrogens differs from that reported previously, and may account for the disparity between our results and those previously published.[14,15] Proper assignment of the pro-*R* and pro-*S* hydrogens is critical to the determination of stereochemistry at C5, as the coupling between these protons and the C5 methyl, as examined by 2D NOESY and ROESY, can then be used to assign the absolute configuration. Our assignments of stereochemistry are completely consistent with distances derived from our computational models of the two diastereomers of SP (S.I.).

Assays of SP repair were conducted at 30°C under anaerobic conditions; details are provided in the Supporting Information. Under the HPLC conditions utilized 5*R*-SP elutes at 19.3 min, 5*S*-SP elutes at 20.3 min, and thymidine elutes at 14.4 min. As can be seen in Fig 2, time-dependent formation of a peak with the retention time of thymidine is observed when 5*R*-SP, but not when 5*S*-SP, is used as a substrate. The identity of this emerging peak as thymidine was confirmed by co-injection with authentic thymidine and by MS analysis of HPLC fractions corresponding to the peak (S.I.). The synthetic SP as well as the SP peaks in Fig 2 (m/z = 485 (SP) and 507 (SP + Na)), as well as the peak labeled T in Fig 2 (m/z = 265 (T + Na)), gave rise to the expected pattern upon MS analysis. MS analysis of the small peaks eluting between 15 and 17 min show them to result from inhomogenous mixture of small peptides, presumably resulting from protein degradation during the sample workup. These assays have been performed 7 different times, on two different protein samples, using two different buffers and two different reducing agents (dithionite or 5-deazariboflavin), and in all cases SPL repaired the 5*R* but not the 5*S*-SP.

Integration of the thymidine and SP peaks in the chromatograms in Fig 2 have allowed us to quantify the rate of SP repair in this system (Fig 2C) as ~0.4 nmol/min/mg of SPL for the *R* isomer, and 0 nmol/min/mg of SPL for the *S* isomer. These rates can be compared to the rate of 0.33 μmol/min/mg when using *B. subtilis* SPL and UV-irradiated plasmid DNA as a substrate.[11] Little additional SP turnover occurs after the four hour time point shown in Fig 2C, likely due to enzyme instability under assay conditions, and/or to enzyme inactivation. The lower rate of turnover for synthetic dinucleoside SP relative to SP generated by UV irradiation of plasmid DNA is not surprising, as the synthetic SP is not constrained to the conformation found in a DNA strand, and also lacks the phosphodiester bridge and oligonucleotide strand that likely contribute considerably to substrate binding interactions in the SPL active site. We have incorporated the protected SP phosphotriester described in the Supporting Information into an oligo, however our initial assays on this indicated that the enzyme cannot repair the di-SEM protected SP analog, Carell has also incorporated a synthetic SP analog into an oligonucleotide strand, however the analog contains numerous protecting groups and no phosphodiester linkage; no assay data on the resulting oligos were presented.[16] We are currently pursuing deprotection of the SP phosphotriesters (S.I.) and incorporation of them into oligonucleotide DNA, in order to produce stereochemically-defined substrates for further assay and mechanistic studies.

The results presented herein provide direct and quantitative evidence for the stereochemical requirements of SP repair by SPL, demonstrating that SPL repairs specifically the 5*R* isomer of SP. The identification of 5*R*-SP as the substrate of SPL supports the premise that SP, like the more familiar cyclobutane thymine dimer, is a result of UV-induced dimerization of *adjacent* thymines in a DNA strand. Furthermore, the observation that only 5*R*-SP is a substrate for SPL is consistent with the expectation, based on the constraints imposed by the DNA double helical structure, that 5*R* is the SP isomer produced *in vivo* upon UV irradiation of bacterial

spore DNA, an expectation supported by a recent report showing that 5*R* SP is the diastereomer produced upon UV irradiation of a TpT dinucleotide.[17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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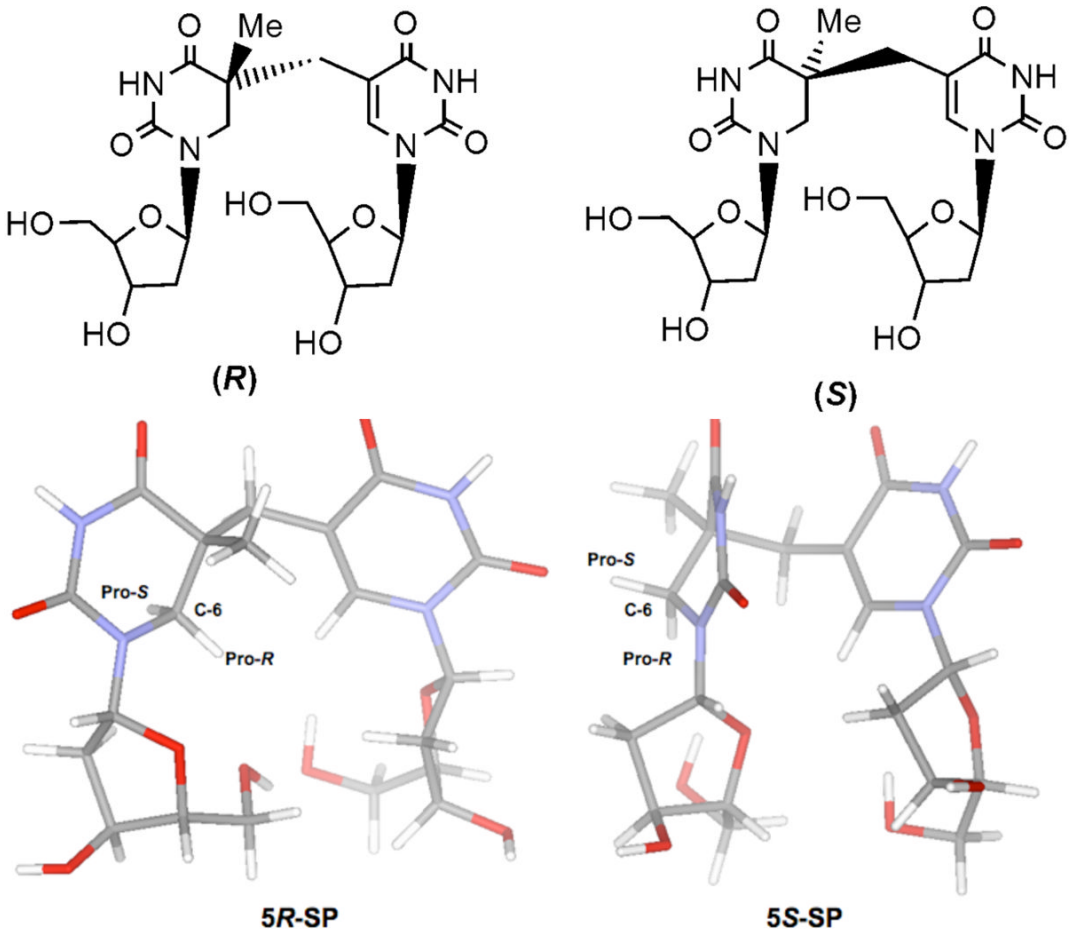


Figure 1.
Chemical drawings (top) and computational models (bottom) for the 5*R* (left) and 5*S* (right) synthetic spore photoproduct analogs lacking a DNA backbone.

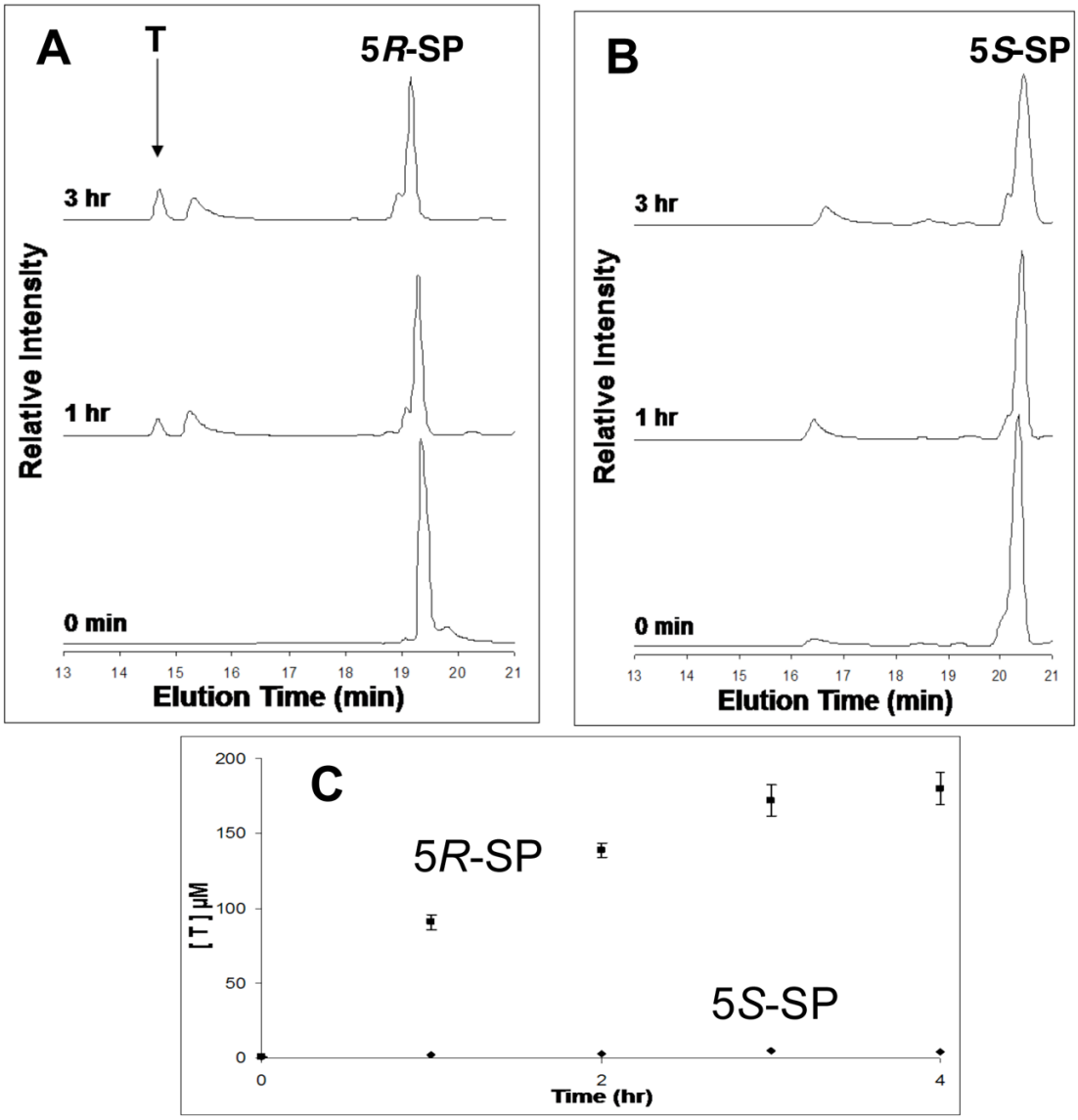
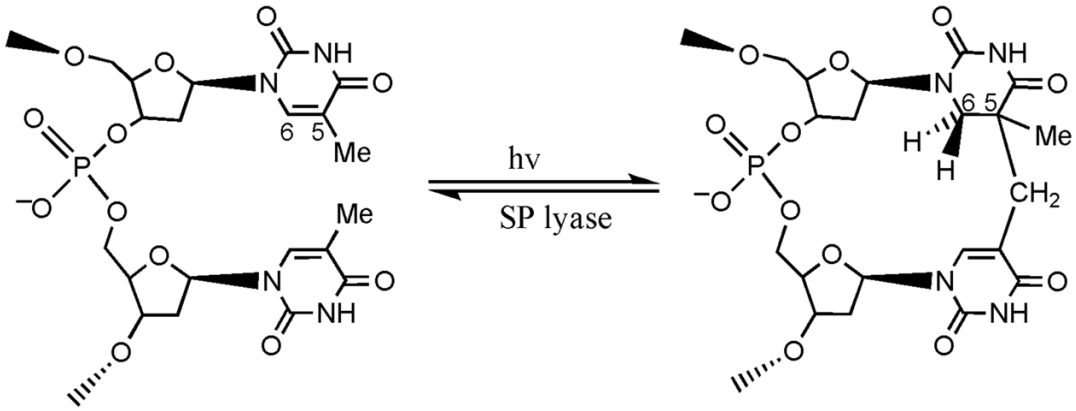


Figure 2.

HPLC chromatograms showing the time-dependent formation of thymidine due to repair of *R*-SP (A) but not *S*-SP (B), upon incubation of 1 mM SP with SPL (50 μM), AdoMet (1 mM), DTT (5 mM), and dithionite (1 mM) in buffer (see S.I.) at 30°C. SP elutes at 19.3 (5*R*) or 20.3 (5*S*) min and thymidine elutes at 14.4 min under these conditions. Integration of the thymidine and SP peaks allowed the quantitation of turnover of each isomer of SP (C).



Scheme 1.
Formation and repair of spore photoproduct.