

Inhibition of translesion DNA polymerase by archaeal reverse gyrase

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ABSTRACT

Reverse gyrase is a unique DNA topoisomerase endowed with ATP-dependent positive supercoiling activity. It is typical of microorganisms living at high temperature and might play a role in maintenance of genome stability and repair. We have identified the translesion DNA polymerase SsoPolY/Dpo4 as one partner of reverse gyrase in the hyperthermophilic archaeon *Sulfolobus solfataricus*. We show here that in cell extracts, PolY and reverse gyrase co-immunoprecipitate with each other and with the single strand binding protein, SSB. The interaction is confirmed *in vitro* by far-western and Surface Plasmon Resonance. In functional assays, reverse gyrase inhibits PolY, but not the *S. solfataricus* B-family DNA polymerase PolB1. Mutational analysis shows that inhibition of PolY activity depends on both ATPase and topoisomerase activities of reverse gyrase, suggesting that the intact positive supercoiling activity is required for PolY inhibition. *In vivo*, reverse gyrase and PolY are degraded after induction of DNA damage. Inhibition by reverse gyrase and degradation might act as a double mechanism to control PolY and prevent its potentially mutagenic activity when undesired. Inhibition of a translesion polymerase by topoisomerase-induced modification of DNA structure may represent a previously unconsidered mechanism of regulation of these two-faced enzymes.

INTRODUCTION

Reverse gyrase is a very unusual DNA topoisomerase, specific of thermophilic microorganisms, which catalyzes positive supercoiling of DNA molecules [for reviews, see refs (1,2)]. It contains a C-terminal topoisomerase

IA domain fused to a N-terminal helicase-like domain, endowed with ATPase activity. The gene for reverse gyrase is present in all hyperthermophiles, either archaea or bacteria, and in some moderately thermophilic bacteria, but has never been found in mesophiles, suggesting a strong link between this enzyme and life at high temperature (3,4). A role for reverse gyrase in adaptation to high temperature was confirmed by the thermosensitive phenotype of a reverse gyrase knock-out mutant (5). Very recently, a reverse gyrase gene found in the genome of *Nautilia profundicola*, a moderately thermophilic bacterium living close to hydrothermal vents, was shown to be transcriptionally induced by 100-fold when the temperature was increased from 45°C to 65°C (6), thus suggesting that reverse gyrase confers a selective advantage to these bacteria facing rapid temperature fluctuations.

The C-terminal domain of reverse gyrase belongs to the universal type IA topoisomerase family, shared by *Bacteria*, *Eucarya* and *Archaea*. The N-terminal domain contains conserved sequence motifs typical of SF2 helicases, including the ATP binding site but, unlike other members of this family, does not show strand separation activity or the ability to move along DNA. The two isolated domains of reverse gyrase behave as independent DNA binding and enzyme modules (7). Whereas the C-terminal domain is clearly responsible for strand passage, the function of the N-terminal domain is poorly understood. The enzyme carries out DNA-dependent ATP hydrolysis, which is essential for positive supercoiling.

Results obtained in our laboratory suggest that reverse gyrase might participate in the cell response to DNA damage. In the crenarchaeon *Sulfolobus solfataricus*, this protein is recruited to DNA *in vivo* after UV irradiation (8), interacts with the single strand binding protein SSB (9) and is specifically degraded after treatment with the alkylating agent methylmethanesulfonate (MMS) (10).

We report here the identification of the translesion (TLS) DNA polymerase IV (Dpo4) as one partner of reverse gyrase in *S. solfataricus*. TLS polymerases belong

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to the recently categorized Y-family that has been identified in *Bacteria*, *Eucarya* and *Archaea* (11–14). Y family DNA polymerases are characterized by low fidelity, lack of a proofreading exonuclease activity and ability to bypass normally replication-blocking lesions. They play essential role in response to DNA damage, but also hold high mutagenic potential. The DinB-like polymerases are taxonomically the most widespread of the Y-family polymerase subgroups. *Escherichia coli* DinB is induced as part of the SOS stress-response system, but might also play some additional physiological role (15). *Sulfolobus solfataricus* Dpo4/SsoPolY (hereafter called PolY), the only Y-family DNA polymerase of this organism, is a direct orthologue of *E. coli* DinB (16–18). It is able to bypass a number of different DNA lesions, such as oxidized and deaminated bases (19–21), N(2)-alkylguanine adducts (22), and others. It interacts with the sliding clamp PCNA (23) and is stimulated by PCNA and the replication factor RFC (24).

We show here that *S. solfataricus* reverse gyrase specifically interacts with PolY *in vitro* and in cell extracts. In primer extension assays, reverse gyrase inhibits the DNA polymerization activity of PolY, and mutational analysis shows that this inhibition is strictly dependent on integrity of reverse gyrase ATPase and topoisomerase activities. *In vivo*, PolY and reverse gyrase are both degraded after treatment of cells with lethal doses of the alkylating agent MMS. The biological meaning of our data and the possible mechanism of the functional interaction are discussed.

MATERIALS AND METHODS

Protein purification

Recombinant His-tagged TopR1 and mutants were expressed in the *E. coli* BL21-AI strain (Stratagene) and purified as described previously (7). PolY was purified as reported (20). Recombinant *S. solfataricus* SSB was purified from *E. coli* transformed with plasmid pET28c-SSB (provided by M. F. White, St Andrews University, UK) using the procedure previously described (25). PolB1 (26) was a gift of F. M. Pisani (Institute of Protein Biochemistry, CNR, Naples, Italy).

Site-directed mutagenesis

The K116A (an alanine residue in place of the histidine in position 116 at the putative ATP-binding site in the N-terminal domain) and the Y965F (an isosteric phenylalanine residue in place of the putative catalytic Tyr 965 in the C-terminal domain) mutations were introduced in pQET7-topR1 using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) and the oligonucleotides shown in Table 1S. The presence of mutations and integrity of the rest of the gene were assessed by DNA sequencing.

Western blots

Total and fractionated extracts were analyzed using the Amersham ECL-Plus kit and a VersaDoc apparatus

(BioRad). Recombinant TopR1 purified from *E. coli* was used to raise custom polyclonal antibodies in rabbit; other antibodies were as follows: anti-PolY (27); anti- β -subunit of *S. solfataricus* pefoldin (28); anti-*S. solfataricus* SSB [gift of M. F. White, St Andrews University, UK (29)].

Far-western

Far-western assays were conducted with modification of the protocol described by Cui *et al.* 2004 (30). Briefly, appropriate amounts of PolY1 were subjected to SDS-PAGE and transferred to Hybond-P PVDF membrane (GE Healthcare). All subsequent steps were performed at 4°C. The membrane was immersed twice in denaturation buffer [6M guanidine HCl in phosphate-buffered saline (PBS)] for 10 min, followed by six times for 10 min in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM DTT. The membrane was blocked in PBS containing 5% powdered milk, 0.1% Tween 20 for 1 h before being incubated 2 h with recombinant TopR1 or TopR1 mutants (0.8 μ g/ml) in PBS supplemented with 0.25% powdered milk, 0.1% Tween 20, 1 mM DTT. The membrane was washed four times for 10 min in PBS containing 0.1% Tween-20, 0.25% powdered milk. The second wash contained 0.0001% glutaraldehyde. Western analysis was then performed to detect the presence of TopR1 using the anti-TopR1 as primary antibody at 1:10 000 dilution. Peroxidase conjugate anti-rabbit (Pierce) was used as the secondary antibody at a 1:50 000 dilution and detected using ECLPlus (GE Healthcare) following the manufacturer's instructions.

Cell growth and extract preparation

Sulfolobus solfataricus P2 cultures were grown and total and fractionated cell extracts were prepared as described (8). UV irradiation and MMS treatment were performed as reported (8,10).

Immunoprecipitations

Ten milligrams of soluble *S. solfataricus* extracts prepared as described (8) were incubated with 15 μ l of a polyclonal antibodies raised in rabbit against recombinant TopR1 or PolY in dilution buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, final volume, 1.5 ml) for 3 h at 4°C with shaking. One hundred and fifty microliters of Protein A-agarose (Roche Applied Science) were added and incubation continued overnight at 4°C with shaking. This solution was centrifuged at 12 000g for 30 s and the beads were washed three times for 20 min at 4°C with 1 ml of wash buffer 1 (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40) and once with 1 ml of wash buffer 2 (25 mM Tris-HCl pH 7.5). The beads were resuspended in 100 μ l of SDS-PAGE loading buffer and heated to 100°C for 5 min. The beads were removed by centrifugation at 12 000 g for 30 s and suitable aliquots of the supernatant were analyzed by western blot.

Positive supercoiling assays

Standard assays were performed as reported (7,9) using plasmid pQE31 (Qiagen) as substrate; products were

separated by two-dimensional agarose gel electrophoresis with ethidium bromide (0.01 µg/ml) in the second dimension. Ethidium bromide-stained gels were analyzed and quantified with a Chemi-doc apparatus and the QuantityOne software (BioRad). Quantitations were performed as reported (9). Briefly, the intensity of each band was experimentally determined and used to calculate the amount of total DNA in each reaction and the relative abundance of each topoisomer. The specific linking difference (σ) was determined using the equation $\sigma = \Delta Lk/Lk_0$. σ values of the topoisomers whose intensity was >30% of the intensity of the most intense topoisomer were considered for the calculation of the mean σ value.

Measurement of the molecular interaction by SPR spectroscopy

Physical interaction was analyzed by the surface plasmon resonance technology (SPR) with a BIAcore 2000 instrument (GE Healthcare). Total 0.6 µmol TopR1, Cter or Nter in 10 mM acetate buffer pH 4.0 were immobilized on a CM5 sensor chip by the Amine Coupling Kit, according the manufacturer's instructions, giving a final amount of immobilization ranged from 4500 to 10000 RU. All the experiments were performed at 25°C in HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% V/V Surfactant P20, pH 7.4). Measurements were performed subtracting the value of the free channel on the sensor chip to eliminate any buffer bulk effect.

ATPase assays

ATPase assays were performed as previously reported (7). Reaction mixtures contained 0.32 nM of heat-denatured phage λ DNA; incubations were performed for 10 min.

Quantitative RT-PCR

Reactions were performed as previously described (10), using M-MLV Reverse Transcriptase and random hexamers (Ambion) to obtain total cDNA, which was then amplified in a BioRad LightCycler using the DyNAmo HS Syber Green qPCR Kit (Finnzymes). Oligonucleotides used for amplification are listed in Table 1S. The expression values of each gene were normalized to the values determined for the 16S rRNA gene. Data reported are from two independent RNA preparations; each cDNA was then used for two independent amplifications and, in each amplification, samples were in triplicate.

Primer extension assay

The substrate used for all primer extension assays (oligoY) was obtained by annealing the 5'-³²P labelled primer (5'-T TAATCCTAATTCGATCGCC-3') to the partially complementary template having the following sequence:

5'-GGACTGTACGTTAAGGCGATCGAATTAGGATTAA-3'.

Standard PolY DNA polymerase reactions (10 µl) were performed as previously described (19) in 40 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 10 mM DTT, 2.5% glycerol,

0.25 mM or 1 mM dNTPs (each), 20 nM annealed 5'-P³²-primer/template. PolB1 was assayed in 30 mM potassium phosphate buffer (pH 7.4) 7.5 mM MgCl₂, 1.25 mM β -mercaptoethanol 5% glycerol, 0.25 or 1 mM dNTPs, 20 nM oligo Y (20). Whenever different enzyme amounts were used, total protein concentration was corrected with BSA. PolY1 or PolB1 were added at 50 nM concentration; TopR1, TopR1 mutants or SSB were added at the indicated concentrations. The reactions were incubated at 55°C for 10 min and terminated by adding 1 volume of stop solution (98% formamide, 10 mM EDTA, 20 mg/ml blue dextran). The products were resolved by electrophoresis in 15% denaturing polyacrylamide/8 M Urea gel and visualized using a Storm Phospho-Imager (Molecular Dynamics, GE Healthcare, Buckinghamshire, UK).

RESULTS

Physical interaction of TopR1 with PolY

Sulfolobus solfataricus genome encodes two reverse gyrase isoforms, named TopR1 and TopR2, both expressed *in vivo* (7,31) and showing the same positive supercoiling activity *in vitro* (MC and AV, unpublished results). Polyclonal antibodies raised against recombinant TopR1 recognized both recombinant proteins and a single band in *S. solfataricus* cell extracts (data not shown).

In order to identify proteins interacting with reverse gyrase, we carried out co-immunoprecipitation experiments from *S. solfataricus* soluble extracts using anti-TopR1 antibodies. Silver staining of gels showed a discrete number of protein bands in immunoprecipitations (data not shown). Western blots carried out with antibodies directed against PolY identified a protein band specifically co-immunoprecipitating with reverse gyrase, but not present in controls with pre-immune sera (Figure 1A). In a reciprocal experiment, an antibody directed against PolY co-immunoprecipitated reverse gyrase from cell extracts, but not controls (Figure 1B). It has been previously shown that the *S. solfataricus* single strand binding protein SSB interacts with reverse gyrase (9,32); we thus tested whether it could also immunoprecipitate with PolY. As shown in Figure 1B, this was the case, thus suggesting that, in cell extracts, reverse gyrase, PolY and SSB are present in binary and/or ternary complexes. As a specificity control, we used an antibody directed against the β -subunit of the chaperone prefoldin (28), a protein not involved directly in DNA metabolism, which revealed no bands immunoprecipitating with PolY.

The physical interaction of reverse gyrase with PolY was confirmed by *in vitro* techniques using the purified recombinant proteins. In Far-western experiments, filter-blotted PolY specifically interacted with TopR1 and with its C-terminal domain, but not with the N-terminal domain or BSA (Figure 1C). The interaction was further characterized by Surface Plasmon Resonance (SPR) analysis. TopR1 or its separate domains were immobilized onto a sensor chip and various concentrations of PolY or other analytes were passed over. PolY interacted with both TopR1 and Cter with a relative binding affinity (K_D)

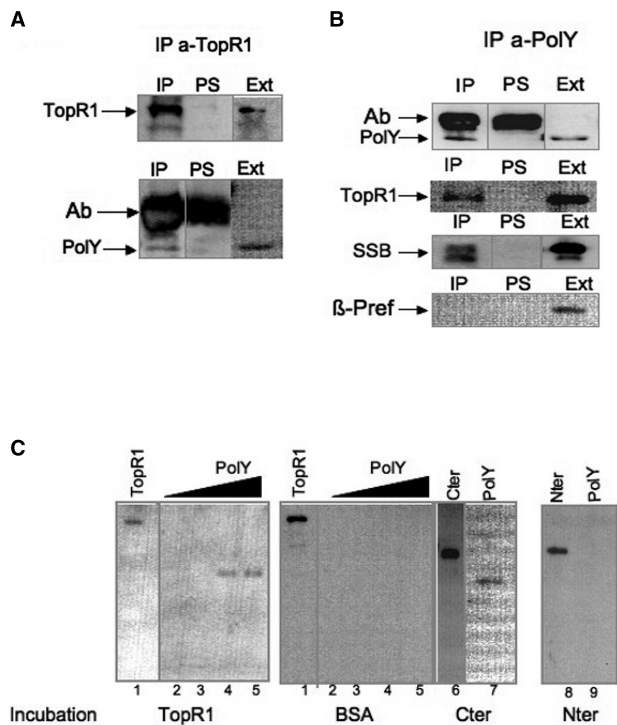


Figure 1. Physical interaction of reverse gyrase and PolY. (A and B) Co-immunoprecipitation analysis. Polyclonal antibodies against TopR1 (A) or PolY (B) or the pre-immune sera (PS) were used to immunoprecipitate *S. solfataricus* soluble cell extracts. Blots were probed with polyclonal antibodies directed against the indicated proteins. IP, immunoprecipitations; Ab, antibodies; Ext, input soluble extracts (20 μ g/lane). (C) Far-western analysis. Increasing amounts of purified recombinant PolY were subjected to SDS-PAGE, transferred to a Hybond-P membrane and then incubated with recombinant TopR1, Cter or Nter; for mock-treatment, filters were incubated with the same concentration of BSA. Western blotting using anti-TopR1 polyclonal antibody was used to detect the presence of proteins on the membrane. Lanes 1, recombinant TopR1 (10 ng); lanes 2–5, 0.1, 0.2, 0.5 and 1.5 μ g of PolY; lane 6 and 8, 50 ng of either Cter or N-ter, respectively; lanes 7 and 9, 1.5 μ g of PolY.

of about 0.01 μ M (Table 1), which was even higher than the K_D value obtained for the interaction between the two domains of reverse gyrase (7). The interaction with the N-terminal domain was much weaker (association constant three orders of magnitude lower). The specificity of the interaction was tested by passing over the chip similar concentrations of BSA or the DNA polymerase SsoPolB1 (26), which interacted with no ligand. We thus conclude that reverse gyrase interacts with PolY both *in vitro* and in cell extracts and the interaction is mediated by the C-terminal domain of reverse gyrase.

Functional interaction of TopR1 with PolY

To test whether the physical interaction of PolY with reverse gyrase might have any functional effect on the catalyzed reactions, optimal conditions to assay both enzyme activities were set up. The TopR1 ATPase activity at 55°C was about 45% of that observed at the optimal temperature of 80°C (Figure 2A), and the efficiency of positive supercoiling at 55°C, as calculated from the mean σ values of the most abundant topoisomers

Table 1. SPR analysis

Analyte	Ligand		
	TopR1	Nter	Cter
PoIY	1.31×10^{-8}	2.73×10^{-5}	1.22×10^{-8}
PoIB	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$
BSA	$<10^{-2}$	$<10^{-2}$	$<10^{-2}$
Nter	ND	ND	1.32×10^{-7}

TopR1 or its separate domains (ligands) were immobilized onto a CM5 sensor chip and increasing concentrations of the analytes were injected. The analysis was performed at 25°C. Each analyte concentration was injected in duplicate and average increases in RU were plotted. Each sensorgram was corrected by subtracting the reference flow cell sensorgram obtained from a reference flow cell. Numbers indicate the dissociation constant (K_D). ND, not determined. The K_D value for the combination Nter as analyte/Cter as ligand was from Valenti *et al.* (7).

produced (see Materials and Methods), was reduced with respect to that observed at 80°C, but still substantial (Figure 2B). Addition of PolY did not have any effect on the TopR1 positive supercoiling activity (data not shown); in contrast, TopR1 inhibited the PolY elongation activity in a concentration-dependent fashion (Figure 2C). Reverse gyrase is able to hydrolyze dATP as efficiently as ATP (33), raising the possibility that inhibition of PolY activity might be due to nucleotide depletion; however, the experiment shown in Figure 2C suggests that this is not the case. Indeed, inhibition was seen at similar extent regardless of whether 0.25 or 1 mM of each dNTP was used (Figure 2C), and even if 1 mM ATP was added to reactions (data not shown). Under the conditions used, considering a specific activity of TopR1 ATPase activity of 0.35 U/mg at 55°C (Figure 2A), the highest amount of TopR1 used (lanes 3 and 6) should hydrolyze about 0.23 nmol/min of dATP. Thus, the final dATP concentration at the end of reactions should be about 0.77 mM (with initial concentration of 1 mM) or 0.02 mM (with initial concentration of 0.25 mM). The dNTP concentration for correct PolY reaction can be as low as 0.01 mM (34). We thus conclude that inhibition was not due to nucleotide depletion.

Interestingly, when used in a similar assay with the DNA polymerase SsoPolB1, TopR1 only marginally affected PolB activity (Figure 2D). Indeed, although a polymerase stop site 2 nucleotides after the start was seen in the presence of TopR1, most PolB molecules reached the full-length product. In contrast, under the same conditions, PolY was completely arrested, suggesting that the effect of reverse gyrase on PolY activity is specific and might be mediated by the physical interaction between the two proteins.

On substrates carrying UV- or MMS- induced lesions, PolY showed a reduced elongation activity as compared with untreated substrates, and it was further inhibited by TopR1 (Figure 1S-A and B). In addition, when dATP was omitted from reactions, PolY almost completely stopped at the position corresponding to the first A, with only one mismatched nucleotide incorporated with low efficiency; TopR1 completely inhibited this reaction (Figure 1S-C).

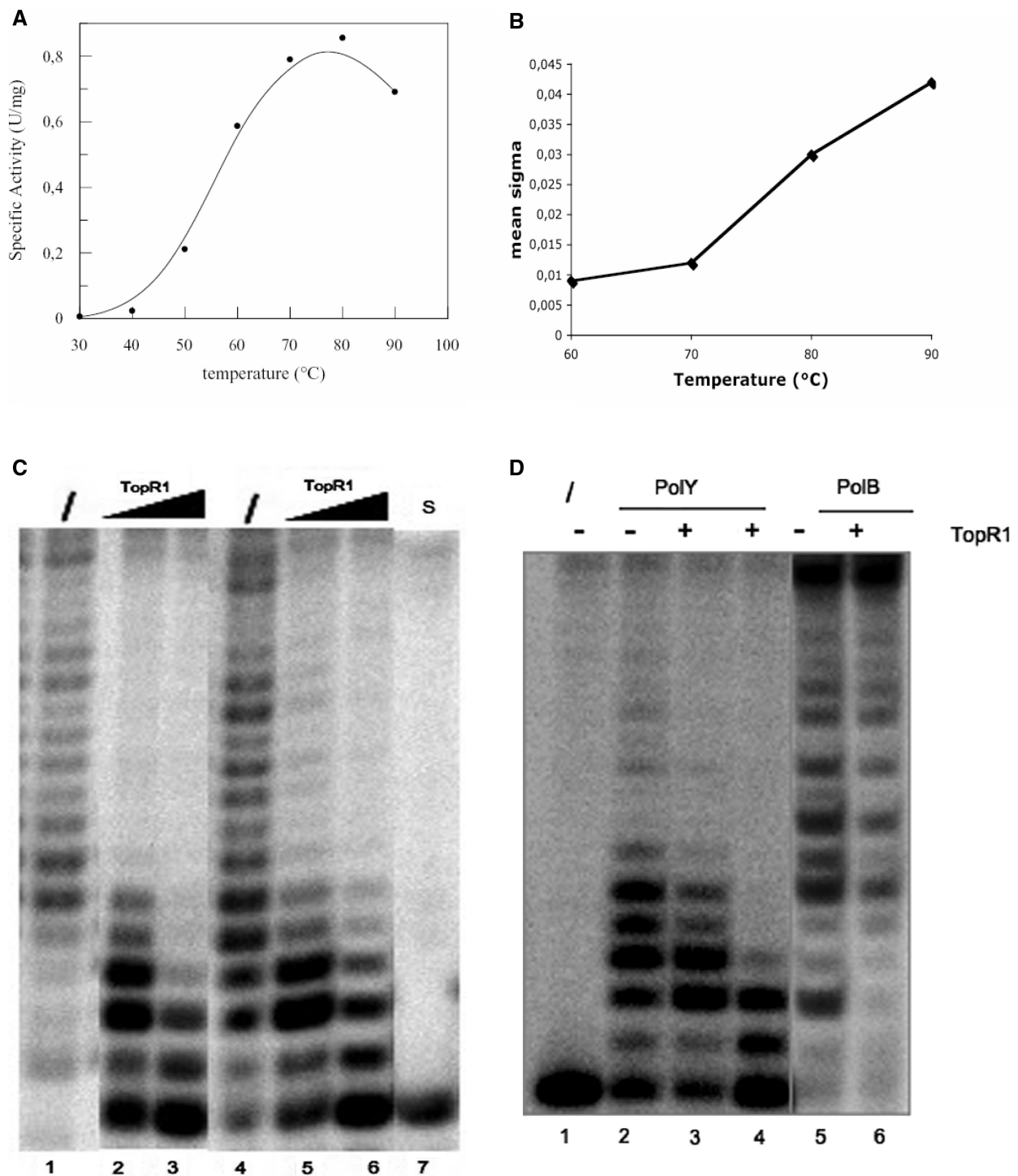


Figure 2. Functional interaction of TopR1 with PolY. (A) Temperature dependence of the ATPase activity. Assays were performed as previously reported (7). Reaction mixtures contained 0.32 nM of heat-denatured phage λ DNA; incubations were performed for 10 min at indicated temperatures. (B) Temperature dependence of TopR1 positive supercoiling activity; the mean specific linking difference (mean σ) was calculated as described in Materials and Methods. Each reaction contained 160 ng of TopR1. Values are mean of three independent experiments. (C) Primer extension assays. PolY (50 nM) was assayed at 55°C for 10 min in the presence of TopR1 at 240 nM (lanes 2 and 5) or 480 nM (lanes 3 and 6); lane 7, substrate alone (S). dNTPs concentration was 0.25 mM in lanes 1–3 and 1 mM in lanes 4–6. Products were separated on 15% denaturing polyacrylamide/urea gel and autoradiographed. (D) Primer extension assays in lanes 2–4 were performed as in lanes 1–3 of Figure 2C; lane 1, no protein; in lanes 5 and 6, SsoPolB1 (50 nM) was incubated either with or without TopR1 (240 nM), as indicated, and incubated for 10 min at 55°C. dNTPs concentration was 0.25 mM.

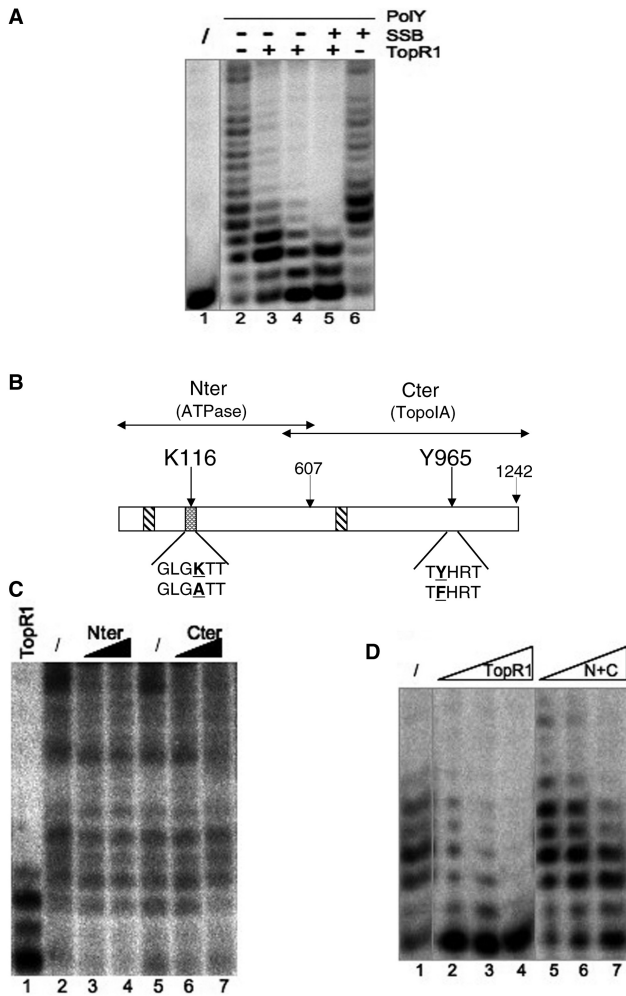


Figure 3. (A) Effect of SSB and reverse gyrase on PolY activity. PolY (50 nM) was assayed in the presence of the indicated proteins at the following concentrations: TopR1, 240 nM (lane 3 and 5) or 480 nM (lanes 4); SSB 600 nM (lanes 5 and 6); lane 1, no protein. (B–D) Characterization of inhibition of PolY activity by TopR1. (B) Schematic diagram of TopR1. Shown are the two separate domains and the mutation introduced at the essential residues K116 in the catalytic site of the ATPase domain and Y965 in the topoisomerase domain; the predicted Zn finger motifs for DNA binding are shown by striped boxes; (C) Primer extension assays. Samples contained PolY (50 nM), alone (lanes 2 and 5) or in combination with one of the following proteins: lane 1, TopR1 (480 nM); lanes 3 and 4, Nter (480 or 960 nM, respectively); lane 6 and 7, Cter (480 or 960 nM, respectively); (D) Primer extension assays. Samples contained PolY (50 nM), alone (lane 1) or in combination with: lanes 2–4, TopR1 (240, 480 or 960 nM); lanes 5–7, Nter and Cter (240, 480 or 960 nM each).

Because of the interaction of both PolY and reverse gyrase with SSB seen in cell extracts, we wanted test the effect of SSB addition. SSB did not influence PolY alone, but further repressed its elongation activity when TopR1 was also present (Figure 3A). The fact that SSB does not inhibit PolY *per se* suggests that its binding to ssDNA does not arrest PolY elongation. *E. coli* SSB was shown to stimulate the TLS polymerase UmuDC, belonging to the PolV family; the different effect of SSB may be due to the different basal activity of the

polymerases: unlike PolY, PolV is inactive by itself and requires auxiliary factors for activity (35).

We have previously shown that the N-terminal (Nter) and C-terminal (Cter) domains of TopR1 behave as independent units, each showing typical DNA binding and enzymatic activity (ATPase and DNA relaxation, respectively; Figure 3B); when combined, they restore the ATP-dependent positive supercoiling activity (7). Interestingly, neither Nter nor Cter had any effect on PolY activity (Figure 3C); however, repression was rescued in the presence of both domains, although with lower efficiency with respect to the full-length enzyme (Figure 3D).

We produced two site-directed mutants of TopR1: K116A, carrying a substitution at the putative ATP binding site in the N-terminal domain, and Y965F, carrying a substitution of the putative catalytic tyrosine in the C-terminal domain (Figure 3B). The biochemical activities of the two mutants were characterized. The topoisomerase activity was tested by 2D agarose electrophoresis (Figure 4A); whereas the wild-type TopR1 showed ATP-dependent positive supercoiling, the K116A mutant was only able to relax DNA, but not to induce positive supercoiling; the Y965F mutant was completely inactive. As for the ATPase activity, the K116A mutant turned out to be inactive, whereas the Y965F mutant displayed kinetic parameters comparable to those of the wild type enzyme (Table 2). We thus conclude that the K116A and Y965F mutations specifically inactivated the ATPase and topoisomerase reactions, respectively; in both mutant enzymes the positive supercoiling activity was impaired. Neither mutant had any effect on the PolY elongation activity, thus suggesting that both ATPase and topoisomerase activities of reverse gyrase are essential for the inhibition to occur (Figure 4B).

Co-regulation of PolY and reverse gyrase by DNA damage

Because we have previously shown that in *S. solfataricus* reverse gyrase is degraded after treatment with the alkylating agent MMS (10), we wondered whether the mutagen could affect PolY level as well. Upon exposure of cultures to MMS, PolY protein level was significantly decreased (Figure 5A). PolY reduction was dependent on both MMS concentration and time of culture treatment and paralleled reverse gyrase reduction.

PolY protein reduction was not due to transcriptional repression, as shown by quantitative RT-PCR analysis of steady-state PolY RNA level, which was even increased upon MMS treatment (Figure 2S).

In extracts prepared from MMS-treated cells, the residual PolY and reverse gyrase content was further reduced upon incubation at 80°C in a time-dependent fashion; in contrast, SSB amount was not affected (Figure 5B); in control extracts, no reduction of either reverse gyrase or PolY content was observed after incubation under the same conditions (Figure 5B). As already shown for reverse gyrase, degradation was partially prevented upon addition of 1,10 phenantroline, an inhibitor of metal-dependent proteases (Figure 5C), thus supporting the involvement of a still unidentified metal-dependent protease.

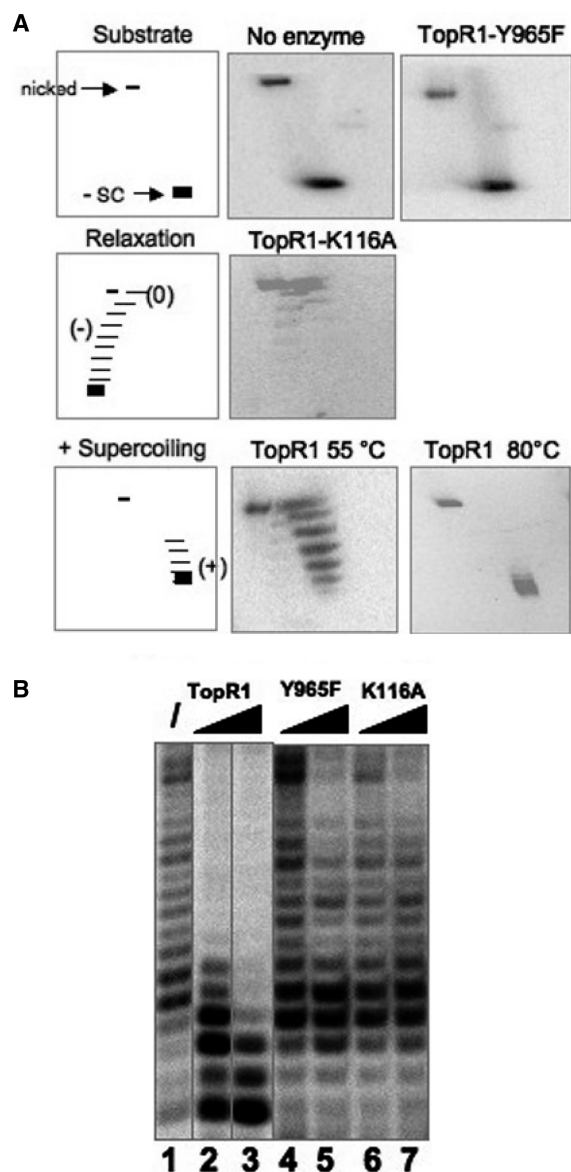


Figure 4. Characterization of TopR1 inhibition activity of PolY. (A) Topoisomerase assays analyzed by 2-D gels. Cartoons show the migration of the negatively supercoiled plasmid substrate (top), a ladder of negative to relaxed (0) topoisomers (middle), and of positive topoisomers (bottom). -SC, negatively supercoiled. Right panels show the results of the experiments performed with 400 ng (final concentration 0.4 nM) of pQE31 plasmid DNA incubated with no enzyme or with indicated enzymes (30 nM) for 10 min at 80°C, except where otherwise indicated. All reactions contained 1 mM ATP. (B) Primer extension assays were performed and analyzed as in Figure 2C. PolY was used at 50 nM alone (lane 1) or in combination with the indicated enzymes. TopR1 and mutants were used at either 240 nM (lanes 2, 4 and 6) or 480 nM (lanes 3, 5 and 7).

Table 2. Steady-state kinetic constants for the ATP hydrolysis at 80°C

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu\text{M}^{-1} \text{s}^{-1}$)
TopR1 wt	77.1 ± 19.4	2.55 ± 0.16	0.033
TopR1 Y965F	256.0 ± 104.6	3.97 ± 0.52	0.016
TopR1 K116A	nd	nd	-

nd: not detectable.

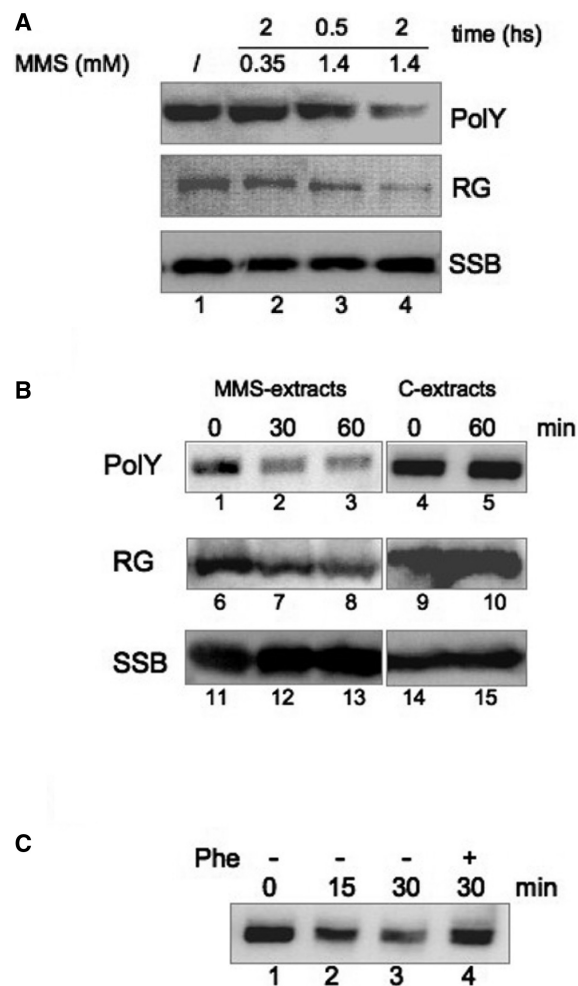


Figure 5. DNA damage-induced regulation of reverse gyrase and PolY levels. (A) Western blots of total soluble cell extracts (15 μg protein/lane) prepared from: controls (lane 1) or cultures treated with MMS at the indicated concentrations for the indicated time spans. The same filter was probed sequentially with the indicated antibodies. (B) Soluble extracts from cultures either mock-treated (C-extracts) or treated for two hours with MMS (1.4 mM), as indicated, were incubated at 80°C for the indicated time spans at the concentration of 10 mg/ml. Because of the different starting reverse gyrase and PolY content in extracts, lanes were loaded with different protein amounts, i.e. lanes 1–3, 15 μg ; lanes 4 and 5, 7.5 μg ; lanes 6–8 and 11–13, 100 μg ; lanes 9, 10 and 14, 15, 30 μg . Filters were probed with the antibodies against the indicated proteins. (C) Soluble extracts from cultures treated for 2 h with MMS (1.4 mM) were incubated at 80°C for the indicated time spans in the absence (lanes 1–3) or presence of 10 mM 1,10-phenanthroline. Filters were probed with the anti-PolY antibody.

DISCUSSION

We have identified the TLS polymerase PolY as one partner of reverse gyrase in *S. solfataricus*. We have shown that the *S. solfataricus* reverse gyrase TopR1 interacts specifically with PolY *in vitro* and the interaction is mediated mainly by the C-terminal domain of TopR1. In cell extracts, PolY and reverse gyrase co-immunoprecipitate with each other and with SSB. In primer extension assays, TopR1 inhibits PolY, but not the *S. solfataricus* DNA polymerase PolB1. Inhibition of PolY activity depends on the intact catalytic activity of TopR1, since deletion mutants containing either the N-terminal or the

C-terminal domain, as well as inactive ATPase or topoisomerase point mutants, were unable to repress PolY activity. These data support the involvement of reverse gyrase in the DNA damage response.

Which activity of TopR1 mediates inhibition? Our data rule out a number of possibilities. First, inhibition is unlikely to depend on competition for DNA; indeed, both Nter and Cter (7) as well as the K116A and Y965F mutants (data not shown) all show high DNA-binding ability. Second, repression does not depend on nucleotide depletion following the ATPase activity of TopR1, since the Y965F mutant shows ATPase activity comparable to the wild-type enzyme, yet has no inhibitory effect. In addition, the inhibition by TopR1 occurs when ATP was added to the reaction or when dNTP were used at concentration 10-fold higher than the Vmax of reverse gyrase ATPase activity. Third, inhibition does not depend on cleavage of the substrate by the TopR1 topoisomerase activity. This conclusion is supported by two observations: (i) the full-length (uncut) primer is seen in all reactions, even at high TopR1 concentrations, and (ii) both the Cter (7) and the K116A (this work) are competent for DNA relaxation, thus for cleavage/religation, yet neither inhibits PolY activity. Our mutational analysis showed that both ATPase and topoisomerase activities of reverse gyrase are essential for repression, suggesting the obvious deduction that it is the positive supercoiling activity that inhibits PolY activity. The observation that addition of SSB, which is known to stimulate reverse gyrase activity (9) further represses PolY when reverse gyrase is present, reinforces this conclusion. Addition of increasing TopR1 concentration results in progressive shortening of PolY elongation products. Thus, the effect of TopR1 is to halt the polymerase at certain sites, progressively reducing the elongation rate. Because PolY can be viewed as a machine moving along DNA, we could describe the action of TopR1 as a brake for the polymerase. The fact that SsPolB1 is only weakly affected by reverse gyrase suggests that either the inhibition is mediated by protein-protein interactions, or that SsPolB1 is insensitive to repression because of its higher processivity.

How could reverse gyrase positive supercoiling activity interfere with PolY activity? PolY inhibition by reverse gyrase might be due, at least in part, by reverse gyrase-induced modification of the substrate. Reverse gyrase is able to bind and cleave the substrate used in these experiments with high efficiency (7 and data not shown); of course, the activity of a topoisomerase on a linear substrate cannot result in stable topological modifications. Nevertheless, reverse gyrase 'gyration' activity could induce local variation of the helical twist, which might persist as long as the enzyme stays on DNA and might prevent PolY binding/activity. Through its interaction with reverse gyrase, PolY might be tethered to templates being modified by the enzyme in such a way to be unsuitable to elongation. Further investigation is required to address the role of template structure in the TopR1-PolY functional interaction.

We have observed that PolY, as previously shown for reverse gyrase (10) is actively degraded following treatment of *S. solfataricus* cells with MMS. Our previous

and present experiments show co-regulation of the two proteins and evidence of the involvement of a metal-dependent protease; unfortunately, attempts to identify this activity have been unsuccessful. Interestingly, we observed a tight inverse correlation between the concentration of MMS used and PolY and reverse gyrase protein levels, which suggests direct correlation between the number of MMS-induced lesions and reverse gyrase-PolY degradation. However, at moment we cannot establish whether the proteins are degraded independently, together, or degradation of one protein destabilizes the other.

Which is the biological implication of our results? We find that PolY is subjected to dual control: inhibition by reverse gyrase and degradation. In principle, the activity of TLS polymerases, which are potentially highly mutagenic, should be tightly regulated. Regulation of TLS polymerases through modification of DNA structure has never been reported before, but might well be a general mechanism. Many proteins affect DNA structure, inducing either covalent (DNA topoisomerases, recombinases) or conformational modification (DNA binding proteins, helicases, translocases). Tethering TLS polymerases to inaccessible sites might be a strategy to control these 'two-faced Janus' enzymes.

Although degradation of archaeal PolY is a novel finding, proteolysis of other TLS polymerases has been reported. In *E. coli*, DinB protein levels are elevated in the absence of the protease ClpXP (36) and UmuC/UmuD (PolV) is subjected to elaborate regulation by degradation involving both Lon and ClpXP proteases (37–39). In *Caenorhabditis elegans* embryos, the Polη TLS polymerase POLH-1 is degraded by a specific protease after treatment with MMS, and indirect evidence suggest that POLH-1 is degraded after performing its function (40). Together with our findings, these data suggest that controlled proteolysis might be a general mechanism of regulation of TLS polymerases. In the case of *S. solfataricus*, degradation of reverse gyrase might liberate PolY, which might in turn be degraded after performing its function. Alternatively, degradation of the complex is simultaneous and is an apoptotic-like process that occurs in the presence of irreparable lesions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Nadal,M. (2007) Reverse gyrase: an insight into the role of DNA-topoisomerases. *Biochimie*, **89**, 447–455.
- Perugino,G., Valenti,A., D'amaro,A., Rossi,M. and Ciaramella,M. (2009) Reverse gyrase and genome stability in hyperthermophilic organisms. *Biochem. Soc. Trans.*, **37**, 69–73.
- Forterre,P. (2002) A hot story from comparative genomics: reverse gyrase is the only hyperthermophile-specific protein. *Trends Genet.*, **18**, 236–237.
- Brochier-Armanet,C. and Forterre,P. (2006) Widespread distribution of archaeal reverse gyrase in thermophilic bacteria suggest a complex history of vertical inheritance and lateral gene transfers. *Archaea*, **2**, i–xi.
- Atomi,H., Matsumi,R. and Imanaka,T. (2004) Reverse gyrase is not a prerequisite for hyperthermophilic life. *J. Bacteriol.*, **186**, 4829–4833.
- Campbell,B.J., Smith,J.L., Hanson,T.E., Klotz,M.G., Stein,L.Y., Lee,C.K., Wu,D., Robinson,J.M., Khouri,H.M., Eisen,J.A. et al. (2009) Adaptations to submarine hydrothermal environments exemplified by the genome of *Nautilia profundicola*. *PLoS Genet.*, e1000362.
- Valenti,A., Perugino,G., D'Amaro,A., Cacace,A., Napoli,A., Rossi,M. and Ciaramella,M. (2008) Dissection of reverse gyrase activities: insight into the evolution of a thermostable molecular machine. *Nucleic Acids Res.*, **36**, 4587–4597.
- Napoli,A., Valenti,A., Salerno,V., Nadal,M., Garnier,F., Rossi,M. and Ciaramella,M. (2004) Reverse gyrase recruitment to DNA after UV light irradiation in *Sulfolobus solfataricus*. *J. Biol. Chem.*, **279**, 33192–33198.
- Napoli,A., Valenti,A., Salerno,V., Nadal,M., Garnier,F., Rossi,M. and Ciaramella,M. (2005) Functional interaction of reverse gyrase with single-strand binding protein of the archaeon *Sulfolobus*. *Nucleic Acids Res.*, **33**, 564–576.
- Valenti,A., Napoli,A., Ferrara,M.C., Nadal,M., Rossi,M. and Ciaramella,M. (2006) Selective degradation of reverse gyrase and DNA fragmentation induced by alkylating agent in the archaeon *Sulfolobus solfataricus*. *Nucleic Acids Res.*, **34**, 2098–2108.
- Nohmi,T. (2006) Environmental stress and lesion-bypass DNA polymerases. *Annu. Rev. Microbiol.*, **60**, 231–253.
- Broyde,S., Wang,L., Rechkoblit,O., Geacintov,N.E. and Patel,D.J. (2008) Lesion processing: high-fidelity versus lesion-bypass DNA polymerases. *Trends Biochem. Sci.*, **33**, 209–219.
- Lehmann,A.R., Niimi,A., Ogi,T., Brown,S., Sabbioneda,S., Wing,J.F., Kannouche,P.L. and Green,C.M. (2007) Translesion synthesis: Y-family polymerases and the polymerase switch. *DNA Repair*, **6**, 891–899.
- Friedberg,E.C., Lehmann,A.R. and Fuchs,R.P.P. (2005) Trading places: how do DNA polymerases switch during translesion DNA synthesis? *Mol. Cell*, **18**, 499–505.
- Jarosz,D.F., Beuning,P.J., Cohen,S.E. and Walker,G.C. (2007) Y-family DNA polymerases in *Escherichia coli*. *Trends Microbiol.*, **15**, 70–77.
- Ling,H., Boudsocq,F., Woodgate,R. and Yang,W. (2001) Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication. *Cell*, **107**, 91–102.
- Silvian,L.F., Toth,E.A., Pham,P., Goodman,M.F. and Ellenberger,T. (2001) Crystal structure of a DinB family error-prone DNA polymerase from *Sulfolobus solfataricus*. *Nat. Struct. Biol.*, **11**, 984–989.
- Zhou,B.L., Pata,J.D. and Steitz,T.A. (2001) Crystal structure of a DinB lesion bypass DNA polymerase catalytic fragment reveals a classic polymerase catalytic domain. *Mol. Cell*, **8**, 427–437.
- Boudsocq,F., Iwai,S., Hanaoka,F. and Woodgate,R. (2001) *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4): an archaeal DinB-like DNA polymerase with lesion-bypass properties akin to eukaryotic poleta. *Nucleic Acids Res.*, **29**, 4607–4616.
- Shimizu,M., Gruz,P., Kamiya,H., Kim,S.R., Pisani,F.M., Masutani,C., Kanke,Y., Harashima,H., Hanaoka,F. and Nohmi,T. (2003) Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases. *EMBO Rep.*, **4**, 269–273.
- Grúz,P., Shimizu,M., Pisani,F.M., De Felice,M., Kanke,Y. and Nohmi,T. (2003) Processing of DNA lesions by archaeal DNA polymerases from *Sulfolobus solfataricus*. *Nucleic Acids Res.*, **31**, 4024–4030.
- Zhang,H., Eoff,R.L., Kozekov,I.D., Rizzo,C.J., Egli,M. and Guengerich,F.P. (2009) Versatility of Y-family *Sulfolobus solfataricus* DNA Polymerase Dpo4 in Translesion Synthesis Past Bulky N2-Alkylguanine Adducts. *J. Biol. Chem.*, **6**, 3563–3576.
- Xing,G., Kirouac,K., Shin,Y.J., Bell,S.D. and Ling,H. (2009) Structural insight into recruitment of translesion DNA polymerase Dpo4 to sliding clamp PCNA. *Mol. Microbiol.*, **71**, 678–691.
- Grúz,P., Pisani,F.M., Shimizu,M., Yamada,M., Hayashi,I., Morikawa,K. and Nohmi,T. (2001) Synthetic activity of Sso DNA polymerase Y1, an archaeal DinB-like DNA polymerase, is stimulated by processivity factors proliferating cell nuclear antigen and replication factor C. *J. Biol. Chem.*, **276**, 47394–473401.
- Wadsworth,R.I. and White,M.F. (2001) Identification and properties of the crenarchaeal single-stranded DNA binding protein from *Sulfolobus solfataricus*. *Nucleic Acids Res.*, **29**, 914–920.
- Pisani,F.M., De Martino,C. and Rossi,M. (1992) A DNA polymerase from the archaeon *Sulfolobus solfataricus* shows sequence similarity to family B DNA polymerases. *Nucleic Acids Res.*, **20**, 2711–2716.
- De Felice,M., Medagli,B., Esposito,L., De Falco,M., Pucci,B., Rossi,M., Grúz,P., Nohmi,T. and Pisani,F.M. (2007) Biochemical evidence of a physical interaction between *Sulfolobus solfataricus* B-family and Y-family DNA polymerases. *Extremophiles*, **11**, 277–282.
- D'Amaro,A., Valenti,A., Napoli,A., Rossi,M. and Ciaramella (2008) The prefoldin of the crenarchaeon *Sulfolobus solfataricus*. *Protein Peptide Lett.*, **15**, 1055–1062.
- Richard,D.J., Bell,S.D. and White,M.F. (2004) Physical and functional interaction of the archaeal single-stranded DNA-binding protein SSB with RNA polymerase. *Nucleic Acids Res.*, **32**, 1065–10674.
- Cui,S., Arosio,D., Doherty,K.M., Brosh,R.M. Jr., Falaschi,A. and Vindigni,A (2004) Analysis of the unwinding activity of the dimeric RECQ1 helicase in the presence of human replication protein A. *Nucleic Acids Res.*, **32**, 2158–2170.
- Garnier,F. and Nadal,M.T. (2008) ranscriptional analysis of the two reverse gyrase encoding genes of *Sulfolobus solfataricus* P2 in relation to the growth phases and temperature conditions. *Extremophiles*, **12**, 799–809.
- Cubeddu,L. and White,M.F. (2005) DNA damage detection by an archaeal single-stranded DNA-binding protein. *J. Mol. Biol.*, **353**, 507–516.
- Rodriguez,A.C. (2002) Studies of a positive supercoiling machine. Nucleotide hydrolysis and a multifunctional “latch” in the mechanism of reverse gyrase. *J. Biol. Chem.*, **277**, 29865–29873.
- Johnson,R.E., Prakash,L. and Prakash,S. (2005) Distinct mechanisms of cis-syn thymine dimer bypass by Dpo4 and DNA polymerase eta. *Proc. Natl Acad. Sci. USA*, **102**, 12359–12364.
- Pham,P., Rangarajan,S., Woodgate,R. and Goodman,M.F. (2001) Roles of DNA polymerases V and II in SOS-induced error-prone and error-free repair in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **Jul 15**, 8350–8354.
- Al Mamun,A.A. and Humayun,M.Z. (2009) Spontaneous mutagenesis is elevated in protease-defective cells. *Mol. Microbiol.*, **71**, 629–639.
- Frank,EG., Ennis,DG., Gonzalez,M., Levine,AS. and Woodgate,R. (1996) Regulation of SOS mutagenesis by proteolysis. *Proc. Natl Acad. Sci. USA*, **Sep 19**, 10291–10296.
- Gonzalez,M., Frank,E.G., Levine,A.S. and Woodgate,R. (1998) Lon-mediated proteolysis of the *Escherichia coli* UmuD mutagenesis protein: in vitro degradation and identification of residues required for proteolysis. *Genes Dev.*, **Dec 24**, 3889–3899.
- Gonzalez,M., Rasulovala,F., Maurizi,M.R. and Woodgate,R. (2000) Subunit-specific degradation of the UmuD/D' heterodimer by the ClpXP protease: the role of trans recognition in UmuD' stability. *EMBO J.*, **19**, 5251–5258.
- Kim,S.H. and Michael,W.M. (2008) Regulated proteolysis of DNA polymerase eta during the DNA-damage response in *C. elegans*. *Mol. Cell*, **32**, 757–766.