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# Biochemical evidence of a physical interaction between *Sulfolobus solfataricus* B-family and Y-family DNA polymerases

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**Abstract** The hyper-thermophilic archaeon *Sulfolobus solfataricus* possesses two functional DNA polymerases belonging to the B-family (Sso DNA pol B1) and to the Y-family (Sso DNA pol Y1). Sso DNA pol B1 recognizes the presence of uracil and hypoxanthine in the template strand and stalls synthesis 3–4 bases upstream of this lesion (''read-ahead'' function). On the other hand, Sso DNA pol Y1 is able to synthesize across these and other lesions on the template strand. Herein we report evidence that Sso DNA pol B1 physically interacts with DNA pol Y1 by surface plasmon resonance measurements and immuno-precipitation experiments. The region of DNA pol B1 responsible for this interaction has been mapped in the central portion of the polypeptide chain (from the amino acid residue 482 to 617), which includes an extended protease hyper-sensitive linker between the N- and C-terminal modules (amino acid residues Asn482-Ala497) and the α-helices forming the ''fingers'' sub-domain (α-helices *R*, *R*′ and *S*). These results have important implications for understanding the polymerase-switching mechanism on the damaged template strand during genome replication in *S. solfataricus*.

**Keywords** DNA replication · Genome stability · DNA polymerase · Translesion synthesis · *Archaea* · *Sulfolobus solfataricus*

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**Abbreviations**

| | |
|---|---|
| Sso: | *Sulfolobus solfataricus* · |
| DNA pol: | DNA polymerase · |
| PAGE: | Polyacrylamide gel electrophoresis · |
| IPTG: | Isopropyl-β-D-thiogalactopyranoside · |
| PMSF: | Phenylmethylsulfonyl fluoride · |
| PVDF: | Poly(vinylidene difluoride) |

## Introduction

Hyper-thermophilic organisms have adopted molecular mechanisms that allow them to maintain genome stability against massive DNA damage caused by exposure to high temperature (Nohmi 2006). Spontaneous deamination, depurination and oxidation of DNA take place at greatly accelerated rate at high temperatures (Lindahl and Nyberg 1974). The hydrolytic deamination of cytosine leads to the formation of uracil in DNA and G:U base pairs result in G:C to A:T transitions in a half of the progeny if not repaired before replication. In addition, deamination of adenine results in formation of adenine that can pair with cytosine, thereby inducing A:T to G:C transitions if not repaired (Lindahl 1993). However, the spontaneous mutation rate in the hyper-thermophilic archaeon *Sulfolobus acidocaldarius* is reported to be similar to that of *Escherichia coli* (Grogan et al. 2001).

Interestingly, B-family DNA polymerases from hyper-thermophilic archaea are able to sense the presence of uracil in the template strand and tightly bind to uracil containing oligonucleotides (Lasken et al. 1996; Greagg et al. 1999). DNA polymerization is stalled when uracil is encountered four bases ahead of the primer-template junction. This ''read-ahead'' function appears to be a peculiar feature of the archaeal B-family DNA pols because B-family DNA polymerases from other organisms (i.e., *E. coli* bacteriophage T4 or yeast and mammals) and thermophilic bacterial A-family enzymes (i.e., *Thermus aquaticus* DNA pol) are able to read through uracil

residues on the template strand without halting DNA synthesis. Furthermore, the B-family DNA pol from *S. solfataricus* (Sso DNA pol B1) was reported to recognize the presence of hypoxanthine in the template, in addition to uracil, and to stall synthesis 3–4 bases upstream of this lesion (Gruz et al. 2003). Stalling of Sso DNA pol B1 at the template uracil has been also visualized by atomic force microscopy (Asami et al. 2006). The "read-ahead" function of the archaeal hyper-thermophilic B-family DNA pols is likely to operate as an additional safeguard mechanism against increased level of deaminated bases in the template DNA strand during genome duplication at high temperature. Analysis of the X-ray crystal structure of some archaeal B-family DNA pols revealed the presence of a special binding pocket for the uracil in the extreme N-terminal portion and site-specific mutagenesis studies confirmed this structural prediction for the B-family DNA pol of *Pyrococcus furiosus* (Fogg et al. 2002).

Interestingly, crenarchaea from the genus *Sulfolobus* possess a DNA pol belonging to the Y-family, in addition to the B-family enzyme (Kulaeva et al. 1996; Gruz et al. 2001). A peculiar feature of Y-family DNA pols is their ability to bypass various lesions in template DNA in an error-prone or error-free manner (Wang 2001; Freidberg et al. 2002). Thus, this family of DNA polymerases plays a critical role in genome stability in bacterial and eukaryotic organisms. Y-family DNA pols from *Sulfolobales* are extremely interesting enzymes since the X-ray structure of two of them (*S. acidocaldarius* and *S. solfataricus*) has been solved providing important structural information about the DNA lesion bypass mechanism (Ling et al. 2001; Silvian et al. 2001; Zhou et al. 2001). The Y-family DNA polymerase from *S. solfataricus* (Sso DNA pol Y1) has been reported to bypass a variety of DNA lesions (Gruz et al. 2001) and to catalyze erroneous incorporation of oxidized dNTPs (such as 8-OH-dGTP and 2-OH-dATP) into nascent DNA (Shimizu et al. 2003).

Herein we report evidence that Sso DNA pol B1 physically interacts with DNA pol Y1 by surface plasmon resonance measurements and immuno-precipitation experiments. The region responsible for this interaction has been mapped in the central portion of DNA pol B1 polypeptide chain (from the amino acid residue 482 to 617). The results of this analysis have important implications for understanding genome stability mechanisms in the hyper-thermophilic crenarchaeon *S. solfataricus*.

## Materials and methods

### Proteins

*His*-tagged Sso DNA pol B1 and the DNA pol Y1 were purified from *E. coli* over-expressing strains as described by Lou et al. (2004) and by Shimizu et al. (2003), respectively.

The truncated forms of Sso DNA pol B1 were produced by PCRs using the pET-DNA pol B1 plasmid DNA as the template. To produce the proteins Sso DNA pol B1-721, -617 and -481 the 5′-primer, named Nt-*Bam*, had the following sequence: 5′-GGGTTT GGATCCGAATGACTAAGCAACTTACCTTA-3′, the *Bam*H1 restriction site is underlined; the 3′-primers were respectively: B-721-*Hin*d (5′-TTGGAAGCTTCTATG TTTCAGTCCAAATACACCGTA-3′); B-617-*Hin*d (5′-TTGGAAGCTTCTATCTCTTCTTCACTAACATCC CCTT-3′); B-481-*Hin*d (5′-GGGTTTAAGCTTCTATT TCGCTAAGATTTCTTCCTT-3′). These latter oligonucleotides contain a stop codon immediately upstream the *Hin*dIII restriction site. The PCR amplified DNA fragment were purified, digested with *Bam*HI and *Hin*dIII and cloned into the *E. coli* expression vector pTRC-*His*C (Invitrogen). All the cloned DNA fragments were sequenced to rule out the presence of undesired mutations.

To produce the recombinant proteins *E. coli* competent cells of the strain BL21(DE3) Rosetta (Novagen) were transformed with each expression plasmid. Transformed cells were grown in 0.5 litre of LB medium supplemented with chloramphenicol (at 30 μg/ml) and ampicillin (at 100 μg/ml). When the culture reached an optical density of 0.7 unit at 600 nm expression of the recombinant protein was induced by adding IPTG at 0.2 mM into the medium. Then the bacterial culture was incubated at 37°C for additional 2 h. Cells were harvested by centrifugation at 8,000 rpm for 10 min at 10°C using a GS-3 Sorvall rotor. Cell pellets were stored at −20°C until use.

Pellets of *E. coli* recombinant cells expressing the truncated protein Sso DNA pol B1-481 or -721 were resuspended in 10 ml of Buffer A (20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 50 mM NaCl) containing protease inhibitors (PMSF at 50 μg/ml, benzamidine at 0.2 μg/ml, aprotinin at 1 μg/ml). Cells were lysed by two consecutive passages through a French press apparatus (Aminco Co., Silver Spring, MD) at 1,500 p.s.i.. The cell extract was centrifuged at 30,000 rpm for 30 min at 10°C in a Beckman 70.0 Ti rotor. The supernatant was incubated at 70°C per 15 min and transferred into ice per 10 min. The heat-treated samples were centrifuged at 30,000 rpm for 30 min at 10°C in a Beckman 70.0 Ti rotor. The supernatants, filtered through a 0.22 μm filter, were loaded onto a $Ni^{2+}$-NTA chelate agarose super-flow column (Qiagen) equilibrated in Buffer A. Bound proteins were eluted by a stepwise gradient of imidazole (10–500 mM) in Buffer A containing 20% glycerol. Collected fraction (1 ml) were analysed by SDS-PAGE and those containing the recombinant protein were pooled and dialysed against Buffer B (20 mM Tris–HCl, pH 8.0, 1 mM DTT, 200 mM NaCl) overnight at 10°C. The sample was concentrated and stored at −20°C.

Pellets of *E. coli* recombinant cells expressing the truncated protein Sso DNA pol B1-617 were treated as described before. The cell extract was incubated for 10 min at 65°C and then transferred to ice for 10 min. The sample was centrifuged at 30,000 rpm for 30 min

using a Beckman 70.0 Ti rotor. The supernatant was filtered through a 0.22 μm filter and subjected to anionic-exchange chromatography on a Mono Q HR 10/10 column (Amersham/Pharmacia Biosciences) equilibrated in Buffer A. The column was developed with a linear gradient of NaCl (0–1 M). Collected fractions (1 ml) were analysed by SDS-PAGE and those containing the recombinant protein were pooled. The sample was dialysed against Buffer C (10 mM Tris–HCl, pH 8.5, 2.5 mM $MgCl_2$) overnight at 10°C and loaded onto a Heparin Sepharose column equilibrated in Buffer C. Fractions containing the recombinant protein were pooled and dialysed against Buffer B overnight at 10°C, concentrated and stored at −20°C.

### Surface plasmon resonance measurements

Real-time interactions of Sso DNA pol B1 and DNA pol Y1 were monitored using the surface plasmon resonance biosensor system Biacore 2000 (Biacore). Sso DNA pol B1 was diluted to a concentration of 20 μg/ml in buffer 10 mM sodium acetate pH 3.6 and coupled to the carboxy-methylated dextran modified gold surface of a CM5 sensor chip, according to the manifacturer's instruction manual. Sso DNA pol Y1 was diluted at 40 μg/ml in buffer 10 mM sodium acetate pH 5.0 and immobilised using the same procedure. Under these conditions, surfaces containing densities of about 2,100 resonance units of Sso DNA pol B1 and 7,000 resonance units of Sso DNA pol Y1 were generated. To collect sensorgrams the indicated proteins at various concentrations were passed over the sensor surface at a flow rate of 10 μl/min. Recorded sensorgrams were normalised to a baseline of zero resonance unit and analysed using the BIA Evaluation software.

### Immuno-precipitation experiments

Protein A Sepharose CL-4B resin (250 μg) was re-suspended in Binding Buffer (50 mM Tris–HCl pH 7.0, 40 mM NaCl, 20 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol) and conjugated with anti-Sso DNA pol Y1 antibodies. Mixtures (final volume: 40 μl) were prepared which contained in Binding Buffer: 9 μg of Sso DNA pol B1 and 7 μg of Sso DNA pol Y1, 9 μg of Sso DNA pol B1 alone (negative control experiment), 7 μg of Sso DNA pol Y1 alone (positive control experiment). To each mixture 80 μl of Protein A Sepharose resin conjugated with anti-Sso DNA pol Y1 antibodies were added. The samples were incubated for 1 h at room temperature with gentle shaking. The resin of each mixture was washed with 5 ml of Washing Buffer (50 mM Tris–HCl pH 7.0, 300 mM NaCl, 1 mM $MgCl_2$, 1% Triton-X-100) and then re-suspended in 60 μl of SDS-PAGE Sample Buffer 1× (62 mM Tris–HCl pH 6.8, 1% glycerol, 0.5% SDS, 0.5% 2-mercapto-ethanol, 0.01% blue bromophenol). Samples were run on a 10% polyacrylamide denaturing gel. After the electrophoretic run the gel was transferred to a PVDF membrane, which was cut into two halves: the upper part was analysed using anti-His antibodies conjugated with horseradish peroxidase (Qiagen) and the ECL + system (Amersham/Pharmacia Biosciences). The lower half of the membrane was analysed using anti-Sso DNA polY1 antibodies and the anti-rabbit IgG antibodies conjugated with alkaline phosphatase as the primary and secondary antibody, respectively.

## Results

### Direct physical interaction between Sso DNA pol B1 and DNA pol Y1

The physical interaction between Sso DNA pol B1 and DNA pol Y1 was monitored using the surface plasmon resonance Biacore 2000 system. In an initial set of experiments Sso DNA pol B1 solutions of increasing concentrations were passed over a DNA pol Y1-immobilised CM5 sensor chip. Fig. 1 shows an example of overlaid sensorgrams obtained with four different concentrations of DNA polB1 (from 0.15 to 1.2 μM). The amplitude of the curves is proportional to the concentration of the analyte, suggesting a direct physical association of the two proteins. The dissociation rate of DNA pol B1 was very low and the equilibrium dissociation constant ($K_D$) was in the order of $1 \times 10^{-8}$ M. Evidence for a physical interaction between the two *Sulfolobus* DNA pols was obtained in similar experiments where DNA pol Y1 was used as the analyte and DNA pol B1 as the ligand (data not shown).

The Sso DNA pol B1/DNA pol Y1 interaction was also tested by immuno-precipitation experiments using the purified recombinant proteins. Protein A Sepharose beads conjugated with anti-DNA pol Y1 antibodies were added to mixtures of the two polymerases. As shown in Fig. 2, DNA pol B1 was pulled down with

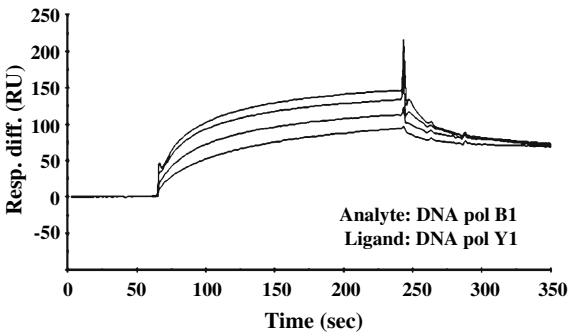


**Fig. 1** Physical interaction between Sso DNA pol B1 and DNA pol Y1 detected by surface plasmon resonance measurements. An overlaid plot of sensorgrams was obtained by fluxing DNA pol B1 at various concentrations over a DNA pol Y1-immobilised sensor chip (*lower to upper curve* DNA pol B1 was at 0.15, 0.3, 0.6 and 1.2 μM, respectively), as described in Materials and methods

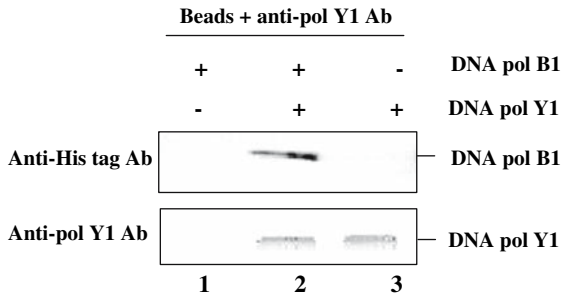


**Fig. 2** Physical interaction between Sso DNA pol B1 and DNA pol Y1 detected by immuno-precipitation experiments. Immuno-precipitation experiments were carried out using Protein A Sepharose beads conjugated with anti-DNA pol Y1 antibodies. The following protein samples were analysed: *lane 1*, Sso DNA pol B1 alone (9 μg, negative control experiment); *lane 2*, a mixture of Sso DNA pol B1 (9 μg) and Sso DNA pol Y1 (7 μg); *lane 3*, Sso DNA pol Y1 alone (7 μg, positive control experiment). After the electrophoretic run the gel was transferred to a PVDF membrane, which was cut into two halves: the upper part was analysed using anti-His antibodies using the ECL+ system to detect the His-tagged Sso DNA pol B1. The lower half of the membrane was analysed using anti-Sso DNA polY1 antibodies and the anti-rabbit IgG antibodies conjugated with alkaline phosphatase as the primary and secondary antibody, respectively

DNA pol Y1 in these experiments confirming that a physical association takes place between the two proteins, as also indicated by the surface plasmon resonance analyses.

## Production of Sso DNA pol B1 truncated forms

In previous studies we demonstrated that the Sso DNA pol B1 possesses a modular organization with regions that are hyper-sensitive to the proteolytic digestion (Pisani and Rossi 1994; Pisani et al. 1996). The resolution of the Sso DNA pol B1 crystallographic structure by X-ray diffraction analysis confirmed the domain organization of the protein (Savino et al. 2004). Based on this knowledge, we designed truncated forms of DNA pol B1 with the aim of identifying the protein region responsible for the physical interaction with DNA pol Y1 (see Fig. 3). The DNA pol B1-721 deleted protein lacks the whole "thumb" sub-domain (residues 721–882) which is responsible for the interaction with the DNA template strand. The truncated form DNA pol B1-617 lacks almost the entire polymerase module, excluding a small portion of the "palm" sub-domain (the beta-sheets 19–21 and the alpha-helix Q) and the "fingers" sub-domain. The truncated protein DNA pol B1-481 consisted of the protein N-terminal half including the uracil-binding pocket and the proof-reading exonuclease domain. These three C-terminally deleted forms of DNA pol B1 were produced in *E. coli* as *His*-tagged proteins using the pTRC-*His* vector system and found to be soluble. Their purification was readily attained by heat-treatment of the cell extracts and chromatographic procedures (see Fig. 3).

## Interaction of the Sso DNA pol B1 truncated forms with the DNA pol Y1

The physical interaction of the Sso DNA pol B1 deleted forms with DNA pol Y1 was probed by surface plasmon resonance measurements. In these experiments solutions of each purified protein of various concentrations were passed over a DNA pol Y1-immobilised CM5 sensor chip. The protein DNA pol B1-481 was unable to interact with DNA pol Y1 suggesting that the C-terminal polymerase domain (residues 482–882) contains the region critical for the interaction with DNA pol Y1.

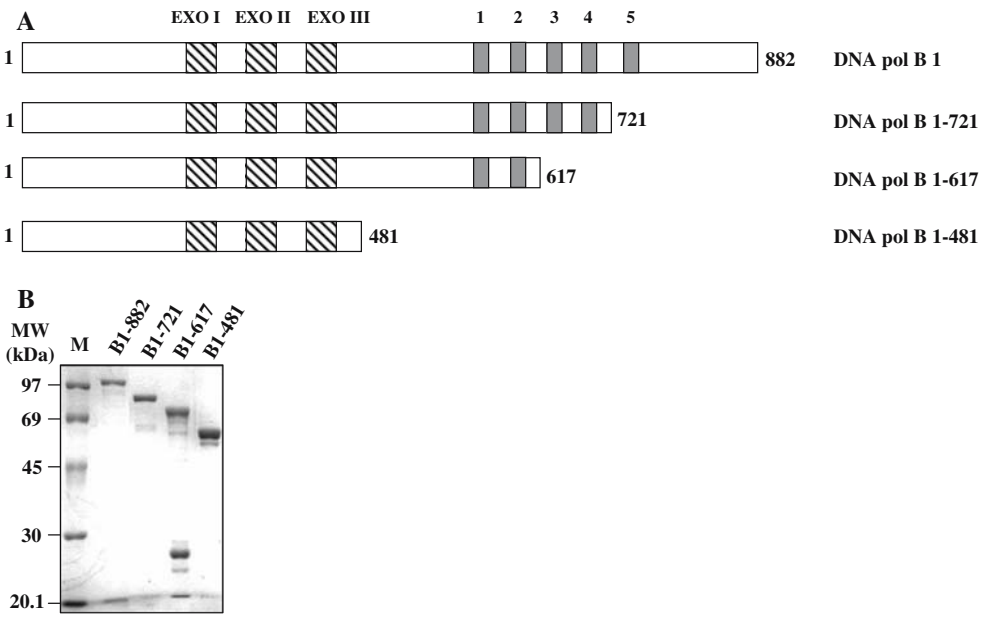


**Fig. 3** Diagrammatic representation of Sso DNA pol B1 deleted forms used in this study. **a** The position of the B Family DNA pols sequence similarity motifs is indicated (Blanco et al. 1991). **b** Electrophoretic analysis of purified DNA pol B1 and its deleted forms (5 μg) on a 10% SDS-polyacrylamide gel and Coomassie Blue staining. The molecular weight of size markers run in the *lane M* are reported on the left part of the panel

Surface plasmon resonance measurements were also carried out using either Sso DNA pol B1-617 or -721 truncated proteins as the analytes. Both these truncated forms were found to physically associate with the DNA pol Y1 with a binding affinity comparable with the full-sized DNA pol B1. As shown in Fig. 4, similar sensorgrams were obtained by fluxing each DNA pol B1 C-terminal deleted form over a DNA pol Y1-immobilised sensor chip. These results suggest that the protein region from residue 482 to 617 is critical for the DNA pol B1/DNA pol Y1 interaction.

## Discussion

The hyper-thermophilic crenarchaeon *S. solfataricus* has two functional DNA polymerases, DNA pol B1 and DNA pol Y1. We previously reported that these DNA pols are expressed at about the same level in *S. solfataricus* cells by quantitative Western blot analysis suggesting that both enzymes play an important role in genome maintenance (Gruz et al. 2003). Sso DNA pol B1 is able to recognize the deaminated bases uracil and hypoxanthine on the template DNA strand and to stop synthesis 3–4 bases upstream of these lesions. In addition, Sso DNA pol B1 also stops nucleotide incorporation 1 base before 8-oxo-guanine. In contrast, Sso DNA pol Y1 belongs to the family of translesion synthesis DNA pols and readily bypasses all these damaged bases in the template strand. Our analysis has revealed that Sso DNA pol B1 and DNA pol Y1 directly interact in vitro, as assessed by surface plasmon resonance measurements and immuno-precipitation experiments. The results of the protein-protein interaction experiments carried out with the DNA pol B1 truncated forms suggest that the region responsible for this physical interaction spans amino acid residues 482–617. This portion of the Sso DNA pol B1 polypeptide chain includes the extended protease hyper-sensitive linker between the N-and C-terminal modules of the enzyme (amino acid residues Asn482-Ala497) and the α-helices forming the "fingers" sub-domain (α-helices *R*, *R′*and *S*; Savino et al. 2004). The extended linker was demonstrated to be highly flexible and exposed to the solvent in a limited proteolysis study (Pisani et al. 1996) and this notion has been subsequently confirmed by the X-ray crystallographic analysis of Sso DNA pol B1 (Savino et al. 2004). In fact, the 3D structure of this region is not traced in the protein structural model because of its poorly or completely undefined electron density due to high conformational mobility. It is tempting to speculate that interaction with DNA pol Y1 could stabilize this linker region and protect it against the protease attack in vivo.

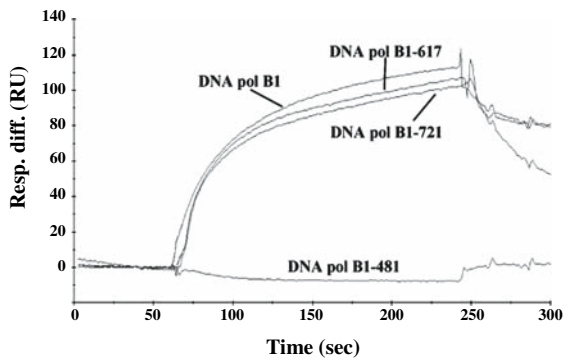


**Fig. 4** Physical interaction of the Sso DNA pol B1 truncated forms with DNA pol Y1 detected by surface plasmon resonance measurements. Plot of sensorgrams obtained by fluxing each indicated protein at 0.3 μM over a DNA pol Y1-immobilised sensor chip, as described in Materials and methods

Mouse and human REV1 subunit of DNA pol ζ were reported to physically interact with the trans lesion synthesis DNA pols ι, η and κ and this interaction was proposed to play a pivotal role in the multi-enzyme, multi-step process of lesion bypass in mammal cells (Guo et al. 2003; Ohashi et al. 2004). In *S. solfataricus* cells, stalling of DNA pol B1 at the template uracil or hypoxanthine may generate single strand gap downstream of the lesion site. Gap filling is likely to be carried out by homologous recombination or translesion synthesis by DNA pol Y1. This latter event requires a switch from DNA pol B1 to DNA pol Y1 at the damaged base. Based on the results of our biochemical analysis, we propose that the physical interaction between Sso DNA pol B1 and DNA pol Y1 could play a role in the polymerase switching mechanism at the lesion site during chromosomal replication. However, we were unable to detect any effect on the uracil-bypass efficiency by Sso DNA pol Y1 in the presence of the full-sized DNA pol B1 or its C-terminal truncated forms. It is quite likely that other factors participate in the translesion synthesis process in *S. solfataricus* cells. After lesion bypass by DNA pol Y1, additional DNA repair enzymes might be recruited at the stalled replication fork, such as DNA glycosylases. These enzymes have been identified in various hyper-thermophilic archaea (Sartori and Jiricny 2003; Chung et al. 2003). It has been shown that the uracil DNA glycosylases physically interacts with the PCNA-like sliding clamp in *Pyrobaculum aerophilum* (Yang et al. 2002) and in *S. solfataricus* (Dionne and Bell 2005). This interaction could be responsible for the recruitment of these and other DNA repair enzymes at the replication fork in vivo. However, our attempts failed to detect a direct physical interaction between Sso DNA pol Y1 and any of the three *S. solfataricus* PCNA-like homologs by surface plasmon resonance experiments suggesting that this interaction is not stable enough to be detected by this technique. Analysis of the physical and/or functional interaction between the *S. solfataricus* DNA pols and the enzymes responsible for repairing deaminated bases in DNA removal will provide insights into genome maintenance mechanisms in this hyper-thermophilic crenarchaeon.

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