Nucleic Acids Research Advance Access published June 22, 2016

Nucleic Acids Research, 2016 **1**
doi: 10.1093/nar/gkw494

MutS regulates access of the error-prone DNA polymerase Pol IV to replication sites: a novel mechanism for maintaining replication fidelity

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Received December 17, 2015; Revised May 4, 2016; Accepted May 20, 2016

ABSTRACT

Translesion DNA polymerases (Pol) function in the bypass of template lesions to relieve stalled replication forks but also display potentially deleterious mutagenic phenotypes that contribute to antibiotic resistance in bacteria and lead to human disease. Effective activity of these enzymes requires association with ring-shaped processivity factors, which dictate their access to sites of DNA synthesis. Here, we show for the first time that the mismatch repair protein MutS plays a role in regulating access of the conserved Y-family Pol IV to replication sites. Our biochemical data reveals that MutS inhibits the interaction of Pol IV with the β clamp processivity factor by competing for binding to the ring. Moreover, the MutS–β clamp association is critical for controlling Pol IV mutagenic replication under normal growth conditions. Thus, our findings reveal important insights into a non-canonical function of MutS in the regulation of a replication activity.

INTRODUCTION

Replication of chromosomal DNA is a highly organized process performed by multi-protein replisome complexes. One of the main replisome components is the replicative DNA polymerase (Pol) that concurrently synthesizes a high-fidelity copy of the leading and lagging strands (1). Pol III holoenzyme (HE) is responsible for the bulk of chromosomal replication in bacteria, whereas the eukaryotic replisome involves Pol ϵ and Pol δ. Replisomes are likely to encounter lesions on the template that generally inhibit DNA synthesis by replicative Pols and are believed to impede fork progression, which constitutes a potentially lethal event (2). Most organisms have evolved to possess specialized Pols that efficiently incorporate nucleotides opposite and past DNA lesions in a process known as translesion synthesis (TLS) (1,3). *Escherichia coli* possess three distinct TLS enzymes: Pol II, Pol IV and Pol V. In mammalian cells, Pol ζ, Pol η, Pol κ and Pol ι, and possibly Pol θ and Pol ν play a role in TLS. Genetic and biochemical analysis demonstrates that most alternative Pols bypass template lesions in either a mutagenic or an error-free manner, depending on the nature of the lesion and the sequence context, but exhibit low fidelity of synthesis when copying undamaged DNA. Thus, although the activities of specialized Pols promote cell survival by allowing the release of replication blocks, their access to sites of DNA synthesis must be tightly controlled to avoid extensive mutagenesis or other potentially deleterious effects on the cell.

Pol IV function has been extensively studied in recent years. This TLS Pol is conserved among all life domains (4) and participates in diverse biological transactions (3). Pol IV catalyzes accurate TLS over N^2-dG adducts and alkylation lesions and the error-prone bypass of oxidized bases (5–7). It has been postulated that Pol IV may also relieve stalled replication forks on undamaged DNA by extending terminal mismatches created but unable to be extended by Pol III HE, or by assisting Pol III HE during synthesis across difficult sequence contexts (8,9). Pol IV shows a high basal concentration in exponentially growing cells compared to expression levels of Pol III HE and other TLS Pols (1,3). However, access of Pol IV to replication sites is limited during normal growth, as suggested by the fact that basal levels of Pol IV do not significantly contribute to spontaneous mutagenesis (5,10). Pol IV expression is upregulated under stressful conditions, thus contributing to mutagenesis that could ultimately result in antibiotic resistance and adapta-

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tion of pathogenic bacteria (5,11–12). Notably, an improved understanding of mechanisms implicated in the regulation of Pol IV access to replication sites will help to develop new therapies to control its function in the acquisition of drug resistance and bacterial pathogenic process.

Multiple mechanisms have been proposed to function in the coordinate regulation of access to DNA replication sites of specialized Pols (1). One mechanism that has gained considerable attention refers to the role played by sliding clamps, the prokaryotic β clamp and the archaeal/eukaryotic proliferating cell nuclear antigen (PCNA). The primary function of ring-shaped sliding clamps is to tether Pols to the template and increase their processivity (13–15). Sliding clamps also function in other genome replication steps, as well as in recombination, repair and cell cycle regulation by interacting with diverse proteins involved in these processes (16,17). For example, sliding clamps associate with the mismatch repair (MMR) proteins bacterial MutS and eukaryotic MutSα (13,18–21). Most of the sliding clamps-binding proteins interact with a hydrophobic cleft on the surface of the ring via a conserved clamp binding motif (CBM) (17,22). In addition, given the homopolymeric structure of sliding clamps, dimeric β clamp and trimeric PCNA contain two or three binding sites, respectively (16,17). Thus, partner proteins can simultaneously interact with or compete for binding the processivity factors. Sliding clamps also play important roles in regulating the action and activity of their partners on DNA. The lesion bypass and mutagenic activities of TLS Pol II, Pol IV and Pol V have been shown to be completely dependent upon the association with β clamp, which targets these Pols to replication sites (23,24).

How Pol IV gains access to β clamp has been extensively studied in Pol III HE replication assays, since it has been proposed that Pol IV activities (TLS and induction of mutagenesis by its overproduction) take place at the replication fork. In TLS reactions reconstituted on DNA templates containing a N^2-dG adduct, processive DNA synthesis by Pol III HE is blocked at the lesion site (25,26). Addition of Pol IV alleviates the block and results in the switching from Pol III HE to Pol IV, which performs the bypass reaction. Pol IV is also able to gain control of β clamp and the primed-DNA when Pol III HE is stalled due to nucleotide omission and, much less efficiently, during active Pol III HE replication of undamaged DNA (26–29). After DNA synthesis by Pol IV, Pol III HE rapidly regains control of the primed-DNA from Pol IV and resumes processive replication. What are the factors controlling the switches between Pol III HE and Pol IV? Structural changes in Pol III HE and/or β clamp, induced by the replication stall or a DNA lesion, may destabilize the association of Pol III HE with β clamp and the primer terminus, and may unmask surfaces of interaction with Pol IV. It has been demonstrated that the Pol exchange involves a specific interaction of Pol IV with a secondary surface of β clamp in addition to the CBM-cleft contact (27,30). A direct interaction of Pol IV with Pol III HE is also likely to contribute to Pol switching (31,32). Thus, a combination of these interactions has been suggested to be necessary to recruit Pol IV to the stalled replisome (32). Interactions between β clamp and the DNA template that it encircles may represent an additional level of regulation of the switching. β clamp–DNA interactions involve the cleft and two additional surfaces of the ring (33), one of which (residues H148-R152) is also required for binding to Pol IV (34). It is expected that β clamp senses the damaged DNA or the replication stall and alters its interaction with the DNA template to enable contact with Pol IV, thereby allowing access of Pol IV to the primer terminus. Notably, other proteins may impose a regulatory control over Pol IV binding to β clamp.

The association between the MMR protein MutS and β clamp has been extensively studied in bacteria and eukaryotes. However, a limited contribution of this interaction in the currently recognized functions of the MMR system, namely the correction of replication errors and inhibition of homologous recombination, has been shown, which raises questions about their functional significance in these organisms (19–21,35). In this work, we present for the first time evidence that MutS from *Pseudomonas aeruginosa* inhibits Pol IV–β clamp interaction by competing for the hydrophobic cleft of the ring *in vitro*. Moreover, MutS–β clamp interaction is critical for controlling of the Pol IV mutagenic activity in exponentially growing *P. aeruginosa* cells. Thus, our findings reveal a key role of MutS in the regulation of the error-prone Pol IV access to replication sites, which corresponds to a novel mechanism for maintaining replication fidelity by MutS.

MATERIALS AND METHODS

Bacterial strains

The experiments were carried out using a *P. aeruginosa* PAO1 strain and its isogenic $mutS^{\beta}$ derivative (19). An SOS non-inducible PAO1 mutant strain, constructed by inserting the non-cleavable *lexA*G86V allele in the chromosome of a *lexA* null mutant, was kindly provided by Dr Pradeep Singh from the University of Washington School of Medicine (36).

Construction of plasmids and chromosomal PAO1 mutant strains, expression and purification of the recombinant proteins, and western blot assays

These experimental procedures are detailed in the Supplementary Data.

Native polyacrylamide gel electrophoresis assays

Native polyacrylamide gel electrophoresis (PAGE) assays were carried out by incubating affinity-purified proteins or the MQSDLFA peptide (Peptide 2.0 Inc) in the presence or absense of DNA oligonucleotides in 20 mM Tris–HCl, pH 7.4, amended with 5 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM NaCl and 100 μg/ml BSA for 25 min at 30°C. The samples were applied to native polyacrylamide gels prepared in Tris-borate buffer and run at 4°C. The gels were Coomassie stained and the protein bands were quantified using the GelPro analyzer software. Additional details are described in the Supplementary Data.

Surface plasmon resonance assays

Surface plasmon resonance (SPR) analysis was performed using a BIAcore T100 instrument (GE Healthcare). Proteins were immobilized on a CM5 chip (GE Healthcare) surface by amine coupling according to the manufacturer's instructions. Soluble proteins were diluted in the Tris 20 mM, pH 7.4, NaCl 25 mM running buffer and injected over chip surfaces at a flow rate of 30 μl min^{-1} for 45 or 90 s at 37°C. Additional details are described in the Supplementary Data.

Dot far-western blotting assays

These assays were performed as previously described (19) with minor modifications as detailed in the Supplementary Data.

Estimation of spontaneous mutation rates and analysis of *nfxB* mutation spectra

Mutation rates were determined by the modified Luria–Delbruck fluctuation test (37). *nfxB* sequence was analyzed from ciprofloxacin resistant colonies (38). Additional details are described in the Supplementary Data.

Transcriptional analysis of the *lexA* promoter

To evaluate SOS-induction, a transcriptional fusion of the *lexA* promoter to the *luxCDABE* reporter operon was inserted into the *P. aeruginosa* chromosome. Luminescence was measured in exponentially growing cells as follow: cultures were diluted to an OD 600 nm of 0.10 and seeded in 96-well plates (100 μl). The 96-well plate was then imaged by integrating luminescence signal for 20 s in a NightOWL LB 983 instrument, and the number of photons emitted per well was quantified using the Berthold WinLigth 32 Software. To evaluate luminescence of colony forming units, plates were imaged using a 1 min exposure time.

N-ethyl-N-nitrosourea sensitivity

Cells were inoculated in LB medium and grown to exponential phase. Cells freshly transformed with the derivatives of p5BAD were cultured in LB medium containing 5 μg/ml gentamicin. Aliquots from successive dilutions were plated onto LB agar supplemented or not with 0.6 mM N-ethyl-N-nitrosourea (ENU), followed by overnight incubation at 37°C. Colony forming units were counted, and survival was determined relative to the LB control, which was set equal to 100%. Transcription from the *lexA* promoter was assayed by monitoring the luminescence of colonies from cells containing the *lexA*::*luxCDABE* reporter.

RESULTS

Pol IV binds to β clamp, albeit with lower affinity than MutS

Pseudomonas aeruginosa MutS (855 amino acids) interacts with β clamp via the ^{816}QSDLF^{820} binding site located at its C terminal domain (19). Change of this motif to ASDAA, obtaining a mutant version denominated MutS$^{\beta}$, abolishes the association with the replicative factor. Interaction between *P. aeruginosa* Pol IV and β clamp has not previously been examined.

Association of Pol IV or MutS with β clamp was initially evaluated by native PAGE (Figure 1A and B). Increasing concentrations of Pol IV or MutS were incubated with a fixed amount of β clamp. The intensity of the band corresponding to free β clamp decreased with increasing Pol IV or MutS concentrations (Supplementary Figure S1A and B). The plot of the β clamp bound fraction as a function of free Pol IV concentrations fitted to a rectangular hyperbola indicating a single binding site ($n = 1.09 \pm 0.06$) on dimeric β clamp (Figure 1A). The sigmoidal-shaped Klotz plot and the linear-shaped Scatchard plot of the same data also suggested a single binding site (data not shown). The K_D value for the Pol IV–β clamp interaction was 6.22 ± 0.51 μM. The MutS–β clamp binding data fitted a sigmoidal curve (Figure 1B). Analysis of this binding data by the Hill equation revealed a positive cooperative interaction, which was confirmed by the concave-downward curve of the Scatchard plot (data not shown). The Hill coefficient was 4.53 ± 1.18 indicating that four MutS molecules bind per β clamp molecule, correlating with the fact that MutS can form tetramers (39). The estimated K_D by Hill plot analysis was 1.66 ± 0.09 μM.

The estimated strengths of Pol IV–β clamp and MutS–β clamp interactions in the native PAGE assays were also observed by SPR (Figure 1C and D). Pol IV specifically bound to β clamp immobilized to a CM5 chip with a K_D = 8.97 μM (Figure 1C). MutS was also able to associate with immobilized β clamp (Supplementary Figure S2), however it was not possible to accurately determine a K_D value for the MutS–β clamp interaction. To further characterize the MutS–β clamp affinity, β clamp was captured using an antibody conjugated to a CM5 chip and then solutions of MutS were made to flow over it. MutS was bound to β clamp 9-fold more strongly than Pol IV did, with K_D = 0.99 μM (Figure 1D). We also examined these interactions by dot far-western blotting analysis (Supplementary Figure S3A). Binding of soluble β clamp to MutS immobilized on nitrocellulose was detected at all tested amounts, and sharply increased between 0.05 and 1.45 pmol of MutS. In contrast, interaction between soluble β clamp and immobilized Pol IV was not observed until Pol IV concentration reached 0.73 pmol. Taken together, our findings clearly indicate that the binding affinity of MutS for β clamp is higher than that of Pol IV for β clamp.

P. aeruginosa Pol IV (349 amino acids) contains the ^{345}QLRLF^{349} hydrophobic cluster at its extreme C-terminus that resembles the consensus β clamp-binding motif QL[S/D]LF identified by bioinformatics analysis of eubacterial β clamp-binding proteins (22). To establish if Pol IV uses this conserved motif to interact with β clamp, a Pol IV mutant version was constructed by deleting the five QLRLF amino acids, herein referred as Pol IV-Δ5, and tested for binding to β clamp. Pol IV-Δ5, as well as MutS$^{\beta}$, failed to associate with β clamp in the native PAGE (Figure 1A and B; Supplementary Figure S1C and D) and the dot far-western blotting assay (Supplementary Figure S3A). Thus, these results indicate that the QLRLF conserved binding motif represents the main site for Pol IV binding to β clamp.

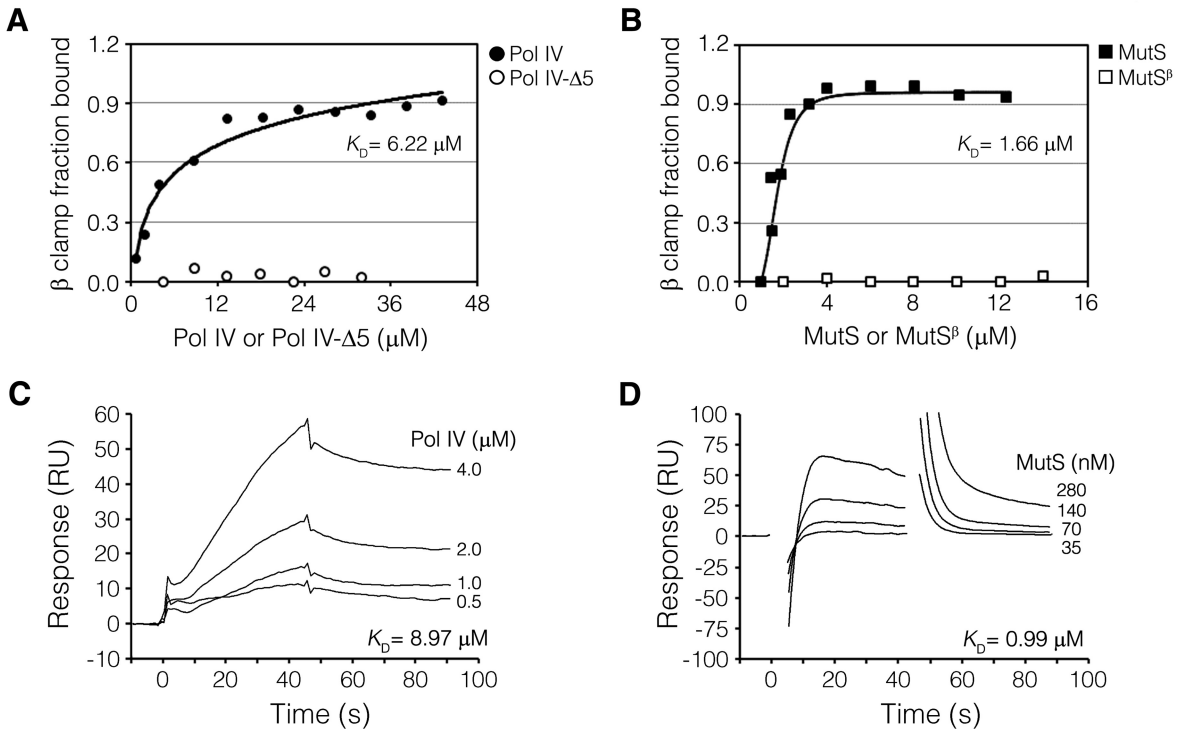

Figure 1. Analysis of Pol IV–β clamp and MutS–β clamp interactions. Saturation binding curves for Pol IV (**A**) and MutS (**B**). Pol IV (1–45 μM), Pol IV-Δ5 (4–32 μM), MutS (1–14 μM) and MutS$^{\beta}$ (2–14 μM) were incubated with a fixed concentration of β clamp (2 μM), and reaction products were analyzed in a native polyacrylamide gel. Saturation binding curves were obtained by plotting the fraction of β clamp bound versus free concentrations of these proteins. A representative experiment is shown ($n = 3$). (**C**) Pol IV–β clamp interaction. β clamp was conjugated to a CM5 chip, followed by injection of Pol IV (0.5–4.0 μM) in SPR-based assays. Kinetic constants for the Pol IV–β clamp interaction were $k_{on} = (5.80 \pm 0.10)\ 10^2\ M^{-1}\ s^{-1}$ and $k_{off} = (5.20 \pm 0.20)\ 10^{-3}\ s^{-1}$. (**D**) MutS–β clamp interaction. β clamp was antibody-captured, followed by injection of MutS (35–280 nM) in SPR-based assays. Kinetics constants for MutS binding to β clamp were $k_{on} = (1.12 \pm 0.08)\ 10^5\ M^{-1}\ s^{-1}$ and $k_{off} = (1.11 \pm 0.04)\ 10^{-1}\ s^{-1}$. Sensograms of MutS displays solvent correction. Representative reference-subtracted curves are shown ($n = 3$).

Finally, as a control, interaction between MutS and Pol IV was examined by SPR (Supplementary Figure S4A and B) and dot far-western blotting (Supplementary Figure S4C–E). No binding was detected between Pol IV and MutS in these conditions.

MutS inhibits Pol IV interaction with β clamp by competing for the protein-binding cleft of the ring

We asked if MutS affects the interaction of Pol IV with β clamp. To examine this issue, Pol IV was immobilized to the CM5 chip and then β clamp alone or pre-incubated with increasing MutS concentrations (30–250 nM) was injected. β clamp interacted with Pol IV, but this association was partially inhibited at 30 nM MutS and abolished by higher MutS concentrations (Figure 2A). Conversely, β clamp binding to Pol IV was not prevented by pre-incubation with 250 nM MutS$^{\beta}$, which did not interact with β clamp in the SPR assays (Supplementary Figure S5). We also examined the formation of the Pol IV–β clamp complex by native PAGE when β clamp, Pol IV and MutS were simultaneously mixed (Figure 2B and Supplementary Figure S1E). MutS, but not MutS$^{\beta}$, hampered the Pol IV–β clamp interaction. At 1 μM MutS, the fraction of β clamp bound to Pol IV was ∼0.45 relative to the control in the absence of MutS, whereas the formation of the Pol IV–β clamp complex was completely abolished at 3 μM MutS. Similar results were obtained in the dot far-western blotting assay (Supplementary Figure S3B). Interaction of soluble β clamp with immobilized Pol IV decreased as increasing amounts of MutS were added into solution. This effect was not detected with the mutant MutS$^{\beta}$ version. We next asked if MutS is able to disrupt the association of Pol IV with β clamp. To further evaluate this question, the Pol IV–β clamp complex was formed and then MutS was added (Figure 2C and Supplementary Figure S1F). The fraction of β clamp bound to Pol IV was reduced following MutS addition as revealed by native PAGE. We only detected a small fraction (∼0.18) of β clamp bound to Pol IV at 3 μM MutS. Addition of the mutant MutS$^{\beta}$ version did not interrupt the Pol IV–β clamp association. Taken together, these results demonstrate that MutS inhibits the formation of the Pol IV–β clamp complex and, is also able to displace Pol IV from the ring.

Finally, we examined whether MutS and Pol IV bind to β clamp at the same site by analyzing if a 7-mer peptide spanning the conserved β clamp binding motif located at the C terminus of MutS (^{815}MQSDLFA^{821}, underlined residues represent the CBM) impairs the Pol IV–β clamp interaction. Addition of this MutS-derived peptide inhibited binding of Pol IV to β clamp in the native PAGE assay. The result, in Figure 3, shows an increase in the intensity of the band corresponding to the β clamp–peptide complex as the peptide

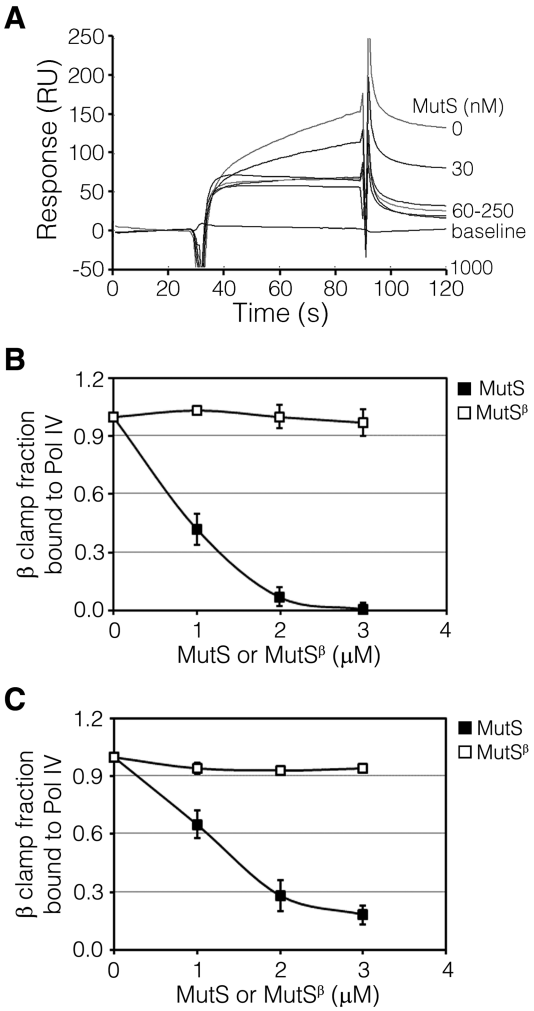

Figure 2. Effect of MutS on the Pol IV–β clamp interaction. (**A**) Interaction of Pol IV with β clamp complexed to MutS. Pol IV was conjugated to a CM5 chip, followed by injection of β clamp (0.25 μM) alone or pre-incubated with MutS (30, 60, 125, 190 and 250 nM) in SPR-based assays. Representative reference-subtracted curves are shown ($n = 3$). (**B**) MutS inhibited Pol IV–β clamp interaction. Pol IV (20.0 μM), β clamp (2.0 μM) and increasing concentrations of MutS or MutS$^{\beta}$ (1.0, 2.0 and 3.0 μM) were simultaneously incubated. (**C**) MutS displaced Pol IV from β clamp. Pol IV (20.0 μM) and β clamp (2.0 μM) were pre-incubated and then MutS or MutS$^{\beta}$ (1.0, 2.0 and 3.0 μM) were added. Reaction products were analyzed by native polyacrylamide gel electrophoresis (PAGE). Band intensities of the Pol IV–β clamp complex were quantified and used to calculate the fraction of β clamp bound to Pol IV taken as 1 the intensity of bands in the control condition corresponding to incubation of Pol IV with β clamp alone. Data represent mean ± standard deviation ($n = 3$).

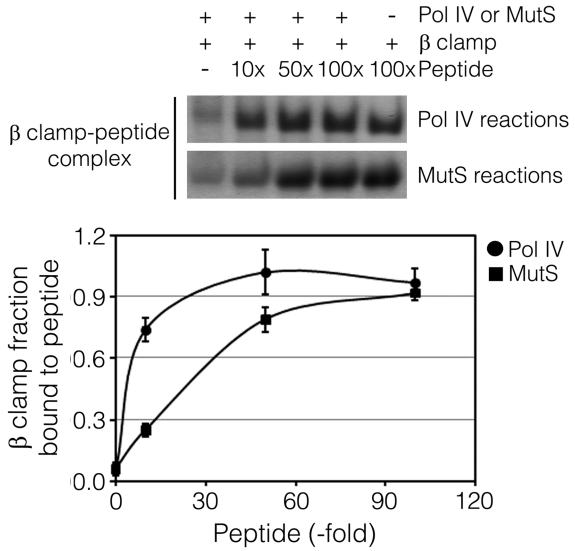

Figure 3. A peptide spanning the β clamp conserved motif of MutS inhibits the interaction of Pol IV with β clamp. Pol IV (20 μM) and MutS (6 μM) were individually incubated with β clamp (2 μM) in the presence of increasing amounts of the MQSDLFA peptide. Concentrations of the peptide were 10-, 50- and 100-fold higher than Pol IV and MutS concentrations. Reaction products were analyzed by a native PAGE. The upper panel shows the band corresponding to the β clamp–peptide complex. The intensity of the β clamp–peptide complex bands was measured, and the fraction of β clamp bound to peptide was calculated taken as 1 the intensity of the band obtained from a reaction wherein β clamp was mixed with the peptide (lower panel). Data represent mean ± standard deviation ($n = 3$).

was titrated into the reaction (Supplementary Figure S1G). As expected, the peptide also impaired the association of MutS with β clamp (Figure 3 and Supplementary Figure S1H). These data further support the conclusion that MutS and Pol IV share a common site on β clamp, namely the hydrophobic cleft between domains II and III of the β ring (40).

DNA structures modulate the ability of MutS to inhibit Pol IV–β clamp interaction

Biochemical studies showed that MutS strongly interacts with heteroduplex DNA and weakly binds homoduplex DNA (19,41). β clamp binds to primed-DNA and homoduplex DNA (33), and Pol IV shows a low affinity for primed-DNA (15). In addition, it has been reported that the presence of DNA substrates affects the affinity between β clamp and its interaction partners (42). Therefore, we examined the effect of different DNA substrates on the affinity of Pol IV–β clamp and MutS–β clamp interactions (Figure 4A). To further investigate this, we mixed these proteins in the presence of single-stranded DNA, homoduplex DNA, GT heteroduplex DNA, primed-DNA or GT primed-DNA. Then, binding was analyzed using native PAGE. The results show that neither DNA substrates had a significant effect on the strength of the Pol IV–β clamp interaction. Strikingly, the MutS–β clamp interaction was enhanced in the presence of the GT primed-DNA, as shown by the 3.5-fold increase in the fraction of bound β clamp compared to the control reaction without DNA. This effect was specific for the GT primed-DNA since the MutS–β clamp interaction did not change when the other DNA substrates were added to the reaction. As a control, and to analyze if the GT primed-DNA favored the MutS–β clamp association by bringing together both proteins onto this DNA structure, we tested the binding between the MutS$^{\beta}$ mutant and β clamp in the presence of this DNA substrate. MutS$^{\beta}$ failed to associate with β clamp under this condition (Supplementary Figure

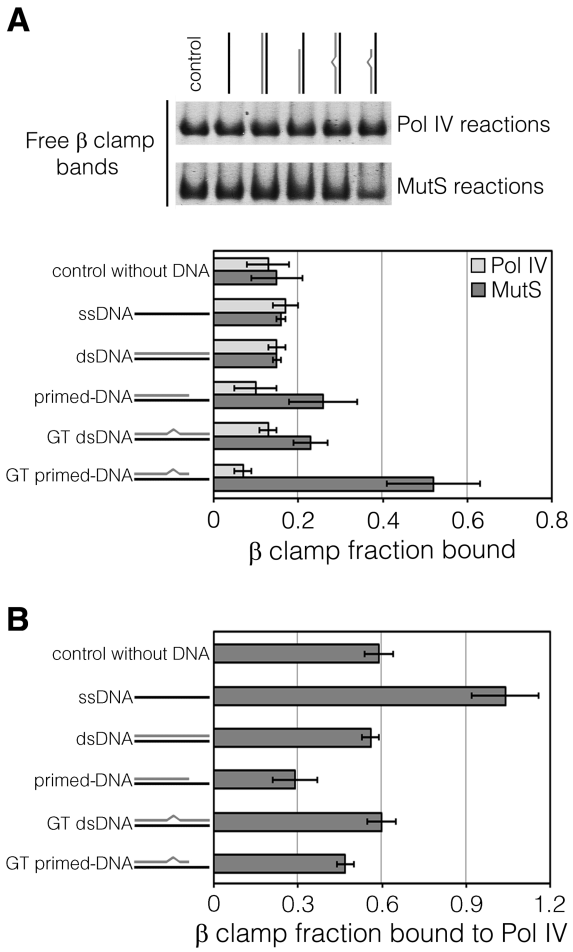

Figure 4. Effect of DNA substrates. (**A**) Pol IV–β clamp and MutS–β clamp interactions. Pol IV (2 μM) and MutS (1 μM) were individually mixed with β clamp (2 μM) in the presence of the DNA substrates (15 μM). Reaction mixtures were analyzed by native PAGE. The intensity of the bands corresponding to free β clamp (upper panel) was quantified and used to calculate the fraction of β clamp bound (lower panel). (**B**) MutS inhibition of the Pol IV–β clamp interaction. Spots of Pol IV (3 pmol) on nitrocellulose membranes were incubated with soluble β clamp (0.25 μM, N-terminally His_6-tagged) mixed with MutS (0.50 μM) and the DNA substrates (15 μM). β clamp binding was detected using a mouse anti-His_6 monoclonal antibody. The intensity of the spots was quantified and used to calculate the fraction of β clamp bound to Pol IV taken as 1 the intensity of spots in the control condition corresponding to soluble β clamp alone. The DNA substrates used were a single-stranded DNA (ssDNA), homoduplex DNA (dsDNA), GT heteroduplex DNA (GT dsDNA), primed-DNA or GT primed-DNA. A control reaction without DNA was also included. Data represent mean ± standard deviation ($n = 3$).

S6), discarding this possibility. These data indicate that a primed-DNA containing a GT mispair specifically modulates the affinity between MutS and β clamp.

We next examined whether the ability of MutS to inhibit Pol IV binding to β clamp is regulated by these DNA structures. This issue was first evaluated by dot far-western blotting (Figure 4B). The fraction of soluble β clamp bound to immobilized Pol IV was reduced when MutS was added into solution (∼0.59 relative to the control in the absence of MutS). The homoduplex DNA and GT heteroduplex DNA had no effect on this MutS ability since similar β clamp fractions were bound to Pol IV (∼0.56 and ∼0.60, respectively). The Pol IV–β clamp association was not inhibited by MutS in the presence of the single-stranded DNA (∼1.04), demonstrating that the capability of MutS for preventing the formation of the Pol IV–β clamp complex is suppressed by this DNA structure. Conversely, the primed-DNA and the GT primed-DNA improved the ability of MutS to inhibit the Pol IV–β clamp interaction as the fraction of β clamp bound to Pol IV was reduced following addition of these DNA substrates (∼0.30 and ∼0.45, respectively). The enhanced capability of MutS for impairing the Pol IV–β clamp interaction in the presence of primed-DNAs was also observed by SPR (Supplementary Figure S7). Reactions of β clamp alone or pre-incubated with the primed-DNA substrates were mixed with MutS (30 or 60 nM) and then injected over Pol IV immobilized to a CM5 chip. β clamp bound to immobilized Pol IV. This interaction was prevented when the processivity factor was mixed with 60 nM MutS, but it was not inhibited at 30 nM MutS. Addition of the primed-DNA or the GT primed-DNA substrates to reactions containing 30 nM MutS abolished β clamp binding to Pol IV. Taken together, these results show that specific DNA structures regulate the effectiveness of MutS as a competitor for the Pol IV–β clamp interaction.

Pol IV strongly contributes to spontaneous mutagenesis in *P. aeruginosa* cells expressing MutS$^{\beta}$

Pol IV overexpression. Pol IV overexpression was shown to result in a weak mutagenesis of chromosomal genes in *E. coli* (9–10,43), *Pseudomonas putida* (11) and *P. aeruginosa* (5). This mutator phenotype is dependent upon the ability of Pol IV to interact with β clamp since this factor targets Pol IV to replication sites, resulting in the low fidelity replication of undamaged DNA (24).

To ascertain whether MutS is able to control interaction between Pol IV and β clamp, and consequently the access of this Pol to replication sites, we examined the effect of Pol IV overexpression on mutagenesis in exponentially growing *P. aeruginosa* PAO1 cells from a *mutS*$^{\beta}$ mutant strain, in which the chromosomal *mutS* allele was replaced by *mutS*$^{\beta}$ that encodes MutS$^{\beta}$ (19), compared with the parental (WT) strain. We tested mutations in different target genes on the *P. aeruginosa* chromosome by measuring mutation rates to resistance to ciprofloxacin (Cip^{r}; *nfxB*, *gyrA* and *parC* are mutated), rifampicin (Rif^{r}; *rpoB* is mutated) and amikacin (Amk^{r}; *mexZ* is mainly mutated) (38,44–46). Overproduction of this Pol was carried out by cloning the *dinB* gene encoding Pol IV into an arabinose inducible plasmid p5BAD (see Supplementary Data). Expression of the ectopic *dinB* gene was detected in *mutS*$^{\beta}$ and WT cells harboring p5BAD-*dinB* after induction with arabinose (Supplementary Figure S8A and B, lines 2–7). This protein was not detected in arabinose-induced cells carrying the empty vector p5BAD (Supplementary Figure S8A and B, line 1). A linear relationship between arabinose concentrations (0.005–0.050%) and Pol IV levels was observed (Supplementary Figure S8E). In addition, similar curves were obtained with the *mutS*$^{\beta}$ and WT strains (Supplementary Figure S8E), indicating that there are not significant differences in the induction of Pol IV in both strains.

Pol IV overexpression from plasmid p5BAD-*dinB* increased mutation rates in the WT and *mutS*$^{\beta}$ strains relative to control cells harboring the empty vector p5BAD (Figure 5A and B; Supplementary Figure S9). It should be noted that equal mutation rates were observed for cells with or without p5BAD (Figure 5B and Supplementary Figure S9C and D). The WT strain displayed 4-, 3- and 2-fold higher mutation rates to Cip^{r}, Rif^{r} and Amk^{r}, respectively, independently of Pol IV expression levels (Figure 5A; Supplementary Figure S9A and B). Thus, although addition of 0.050% arabinose resulted in a 10-fold higher Pol IV expression than that obtained with 0.005% arabinose (Supplementary Figure S8E), there were no significant differences in mutation rates at all arabinose concentrations tested. In the *mutS*$^{\beta}$ strain, mutagenesis was enhanced as Pol IV expression levels increased reaching 22-, 15- and 17-fold higher mutation rates to Cip^{r}, Rif^{r} and Amk^{r} after induction with 0.050% arabinose, respectively (Figure 5A; Supplementary Figure S9A and B). Then, we measured mutation rates in the WT and *mutS*$^{\beta}$ strains containing p5BAD derivatives encoding Pol IV-Δ5 or the catalytic-deficient Pol IV-D8A mutant. Pol IV-D8A had a substitution of the conserved aspartic acid residue to alanine at position 8 in the polymerase catalytic site I, which severely impairs the polymerase activity (5). Introduction of p5BAD plasmids carrying *dinB* alleles coding for these mutant versions failed to enhance the mutation rates after induction with 0.050% arabinose in both strains (Figure 5B; Supplementary Figure S9C and D). Quantification by western blotting revealed that expression levels of both Pol IV mutants were not comparable to that of the wild-type protein at 0.050% arabinose (Supplementary Figure S8A and B, lines 8 and 9). Pol IV-Δ5 was present at 4- and 6-fold higher expression levels and Pol IV-D8A showed a 4- and 10-fold decrease in the WT and *mutS*$^{\beta}$ strains, respectively. It should be noted that these Pol IV-D8A levels were similar to that obtained of wild-type Pol IV at lower arabinose concentrations (0.005 and 0.010%) wherein mutagenesis was increased.

These findings demonstrate that Pol IV ectopically expressed from plasmid is able to introduce mutations on the *P. aeruginosa* chromosome, and this Pol IV-induced mutagenesis is considerably higher in the *mutS*$^{\beta}$ strain to that observed in the WT strain. As previously observed (5,24), both the Pol activity and the interaction with the processivity factor are required to enhance mutagenesis by Pol IV.

Mutation spectra. We next investigated the Pol IV mutational specificity in the *mutS*$^{\beta}$ background compared with the WT background by characterizing the mutation spectra of the reporter gene *nfxB,* which scores a broad range of mutations and allows the analysis of strand bias and preferred sequence contexts of mutation (38). Sequencing of the entire open reading frame and promoter region of *nfxB* from Cip^{r} clones showed that the six base substitutions, 1-bp deletions and insertions and >1-pb deletions were detected within this gene in all PAO1 strains (Supplementary Table S1). When the *nfxB* mutation spectra from the *mutS*$^{\beta}$ and WT strains were compared, there was a significant 2-fold increase in the rate of the AT > CG base substitution in the *mutS*$^{\beta}$ strain (Figure 5C and Supplementary Table S1). To examine if the increase of this transversion is due to the Pol IV activity, we constructed Pol IV-deficient cells by deleting *dinB* from the *mutS*$^{\beta}$ and WT strains. We did not detect any difference in mutation rates to Cip^{r} among the WT, *mutS*$^{\beta}$, *dinB* and *mutS*$^{\beta}$ *dinB* strains (Supplementary Table S1). However, deletion of *dinB* significantly decreased the rate of AT > CG (2-fold), CG > AT (2-fold) and AT > GC (3-fold) in the *mutS*$^{\beta}$ background (*mutS*$^{\beta}$ versus *mutS*$^{\beta}$ *dinB* strains) (Figure 5C and Supplementary Table S1). Notably, Pol IV-deficiency did not decrease the rate of any mutations in the WT background (WT versus *dinB* strains) (Figure 5C and Supplementary Table S1). Pol IV overexpression mainly promoted AT > GC, AT > CG, CG > GC and >1-bp deletions in the *mutS*$^{\beta}$ and WT strains compared with control empty cells (Supplementary Table S1). The rate of these mutations were 13-, 9-, 3- and 6-fold higher in the *mutS*$^{\beta}$ strain relative to that observed in the WT strain (Supplementary Table S1). Finally, we corroborated that these mutations resulted from the Pol IV activity since overexpression of the polymerase activity-deficient Pol IV-D8A mutant did not change the *nfxB* mutation spectra in the WT and *mutS*$^{\beta}$ strains (data not shown).

These data indicate that mutational specificity of *P. aeruginosa* Pol IV is similar to that of *E. coli* Pol IV. Thus, Pol IV overexpression in both bacteria preferentially promotes base substitutions toward GC pairs (43). In addition, and accordingly to the previous data (5,10), Pol IV does not contribute to spontaneous mutagenesis in exponentially growing cells since inactivation of *dinB* does not change the mutation spectra in the WT background. On the other hand, a proportion of spontaneous mutations are generated by this Pol in the *mutS*$^{\beta}$ strain, and overproduction of Pol IV enhances to a greater extent some base substitutions and deletions in this strain.

Strand bias. It was previously observed that overproduction of Pol IV enhances preferentially mutations during lagging strand synthesis in *E. coli* (9). To explore if there is a differential mutator effect of Pol IV in the two replicating strands of the *P. aeruginosa* chromosome, we analyzed each *nfxB* base substitution spectrum for strand bias using the iMARS software (47) (Supplementary Table S2). The mutation spectra from the WT strain showed no strand bias. Overexpression of Pol IV from the p5BAD-*dinB* plasmid caused a strand bias whereas the deficiency of this Pol (*dinB* strain) had no effect in the WT background. A significant strand bias was evident in the mutation spectra from the *mutS*$^{\beta}$ strain. Deletion of *dinB* (*mutS*$^{\beta}$ *dinB* strain) eliminated this strand bias. As expected, *mutS*$^{\beta}$ cells harboring p5BAD-*dinB* retained the strand bias observed in the *mutS*$^{\beta}$ strain. Thus, a preferential mutagenesis on one of the strands of the replication fork was evident in exponentially growing cells of the *mutS*$^{\beta}$ strain, but not in the WT strain, which was dependent on Pol IV.

Sequence context specificity. We found that the GG and GC dinucleotides were most frequently mutated in the *nfxB* base substitution spectra of the *mutS*$^{\beta}$ and WT strains harboring p5BAD-*dinB* using iMARS (data not shown). Both dinucleotides were almost exclusively present in sites undergoing CG > GC transversions, which corresponds to one of the most promoted mutations by Pol IV overexpression

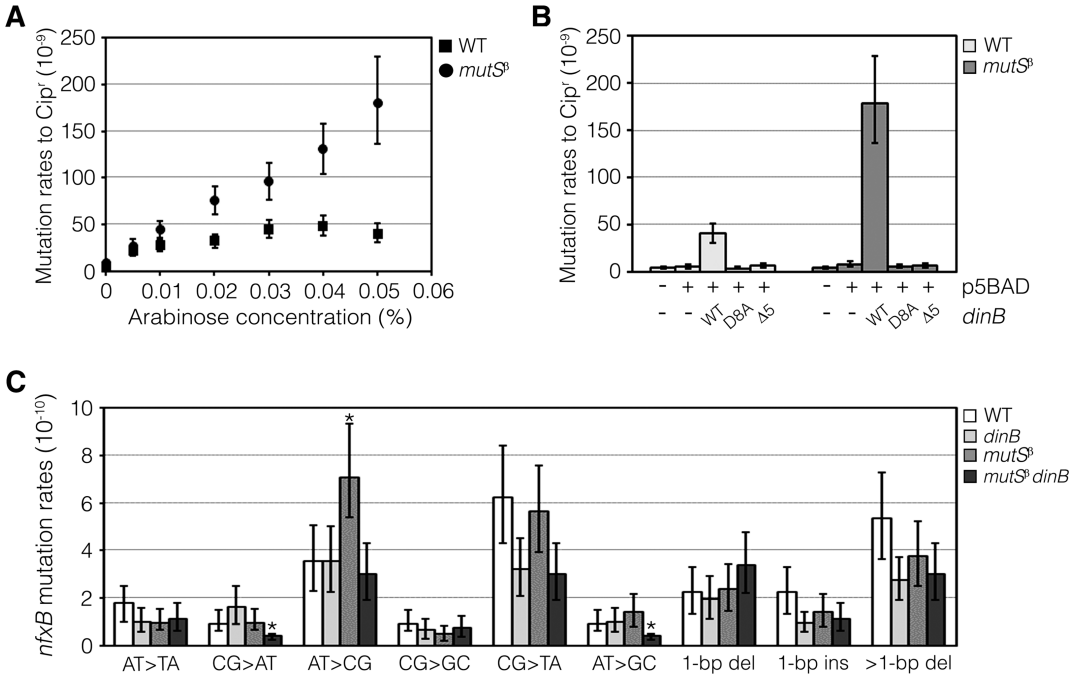

Figure 5. Contribution of Pol IV to spontaneous mutagenesis. (**A** and **B**) Mutation rates to Cip^r per replication and 95% confidence limits were calculated as described in 'Materials and Methods' section. (A) WT and *mutS*^{β} strains carrying p5BAD-*dinB* after induction with different arabinose concentrations (0.005–0.050%). (B) WT and *mutS*^{β} strains without plasmid or carrying p5BAD, p5BAD-*dinB*, p5BAD-*dinB*D8A or p5BAD-*dinB*Δ5 after induction with 0.050% arabinose. (**C**) Rates of each mutation type detected in *nfxB* from Cip^r clones were compared between the WT, *dinB*, *mutS*^{β} and *mutS*^{β} *dinB* strains. Error bars represent the upper and lower 95% confidence intervals. No overlap of 95% confidence intervals indicates statistically significant differences (marked with asterisks in panel C).

(Supplementary Tables S3 and 4). Visual inspection of sequences surrounding the mutated nucleotide revealed that this mutation mainly occurred in the G**G**C trinucleotide (which included the GG and GC dinucleotides; the mutated nucleotide is indicated in bold). In addition, a high proportion of the AT > CG transversions occurred in the G**A**C trinucleotide. Thus, the G**X**C sequence corresponded to a preferred context for Pol IV within *nfxB*. This trinucleotide was detected in 58 and 80% of the base substitutions for the *mutS*^{β} and WT strains containing p5BAD-*dinB*, respectively (Supplementary Tables S3 and 4). In the WT strain, only 3% of base substitutions occurred within G**X**C sequences whereas a major proportion (14%) of these mutations was observed in this sequence context in the *mutS*^{β} strain (Supplementary Tables S5 and 6). In contrast, base substitutions were not present at sites involving the G**X**C sequence in the *dinB* strain (Supplementary Table S7). This trinucleotide was detected in 4% of the base substitutions in the *mutS*^{β} *dinB* strain (Supplementary Table S8).

The sequence specificity of base substitutions induced by *P. aeruginosa* Pol IV resembles to that observed for the *E. coli* enzyme, which preferred the 5′-GX-3′ context where X represents the mutated base within the *c*II and *rpoB* genes (10,43). Moreover, our data show that the G**X**C sequence context is more frequently represented in the mutation spectrum of the *mutS*^{β} strain when compared to that observed in the WT strain, and this depends on the presence of Pol IV.

Pol IV-induced mutagenesis is not controlled by MutS under SOS conditions

Pol IV expression is induced as part of the LexA-dependent SOS regulon in *E. coli* and related bacteria as a response to environmental stress (48). Inactivation of the LexA repressor by a RecA-mediated autocleavage after DNA damage leads to the transcriptional de-repression of the SOS-regulated genes including *dinB* and *lexA*. In *P. aeruginosa*, *dinB* transcription levels are increased upon disruption of *lexA*, confirming that LexA acts to repress *dinB* transcription (5).

We asked if MutS is able to control Pol IV-dependent mutagenesis under SOS-induced conditions. To examine this issue, LexA-deficient derivatives of the WT, *mutS*^{β}, *dinB* and *mutS*^{β} *dinB* strains were constructed by replacing the chromosomal *lexA* gene with an inactive allele (see Supplementary Data). In addition, and in order to measure the relative induction of the SOS gene network, a reporter construction containing the putative promoter region of *lexA* cloned upstream of the *luxCDABE* operon was inserted into the chromosome of strains harboring an active (*lexA*+) or inactive (*lexA*-) allele. An ∼3-fold higher luminescence signal was detected in exponentially growing cultures of the *lexA*- strains relative to that observed in the *lexA*+ strains (Figure 6A), confirming that LexA deficiency induces the SOS response. Then, we measured mutation rates to Cip^r to test mutation levels in cells expressing constitutively the SOS response. *lexA* inactivation significantly increased mutagenesis in the WT and *mutS*^{β} backgrounds (Figure 6B). *lexA* and *mutS*^{β} *lexA* strains showed similar

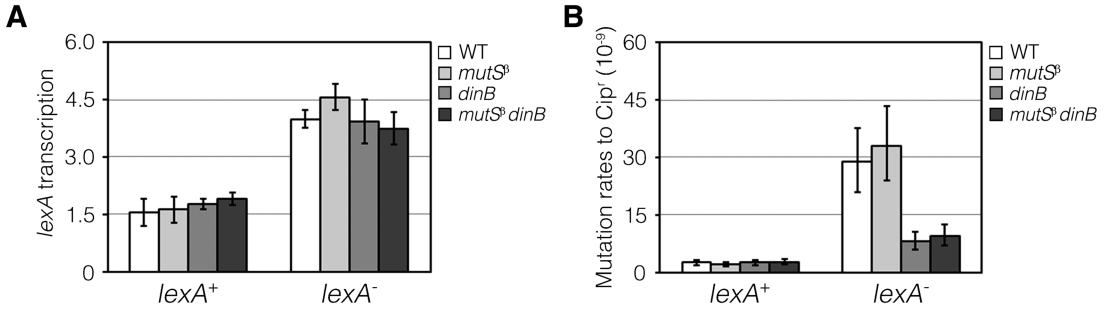

Figure 6. Pol IV-induced mutagenesis under SOS conditions. (**A**) Transcriptional analysis of the *lexA* promoter. Transcription from the *lexA* promoter was assayed by measuring the luminescence in exponentially growing cultures of cells harboring a transcriptional fusion of the *lexA* promoter to the *luxCDABE* reporter operon inserted into the chromosome. Data represent mean ± standard deviation ($n = 3$). (**B**) Mutation rates to Cip^r per replication and 95% confidence limits were calculated as described in 'Materials and Methods' section. Error bars represent the upper and lower 95% confidence intervals. No overlap of 95% confidence intervals indicates statistically significant differences. The strains used were WT, *mutS*$^\beta$, *dinB* and *mutS*$^\beta$ *dinB* harboring an active (*lexA*+) or inactive (*lexA*−) allele in the chromosome. These strains also contained the reporter fusion inserted into the chromosome. It should be noted that equal mutation rates were observed for the *lexA*+ strains with or without the reporter fusion.

mutation rates, which were 11- and 16-fold higher compared to WT and *mutS*$^\beta$, respectively. Deletion of *dinB* considerably decreased mutagenesis under SOS conditions (Figure 6B). *dinB lexA* and *mutS*$^\beta$ *dinB lexA* strains displayed 3.5- and 3.4-fold reduced mutation levels relative to *lexA* and *mutS*$^\beta$ *lexA*, and they exhibited 3.2- and 3.5-fold higher mutation rates compared with *dinB* and *mutS*$^\beta$ *dinB*. These results indicate a key role for Pol IV in SOS-induced mutagenesis, as it was observed in *E. coli* (49). Our data also clearly demonstrate that MutS does not limit Pol IV-dependent mutagenesis under SOS conditions since mutation levels in the WT and *mutS*$^\beta$ backgrounds are similar.

The capability of Pol IV for catalyzing TLS is not affected in the *mutS*$^\beta$ background

Pol IV is involved in accurate bypass of DNA lesions induced by alkylating agents and thus, confers resistance to the killing effect of exogenous alkylating compounds such as ENU (6). To analyze the capacity of Pol IV to participate in TLS events in the WT and *mutS*$^\beta$ backgrounds, we tested the survival of exponentially growing cells after treatment with ENU (Figure 7). Exposure to ENU reduced cell viability of the WT and *mutS*$^\beta$ strains to the same extent. Cell viability was reduced to 15 and 12% of control untreated WT and *mutS*$^\beta$ cells after exposure to ENU, respectively. As expected, Pol IV-deficient cells from the WT and *mutS*$^\beta$ strains were ∼7-fold more sensitive. Pol IV overexpression increases cell survival in both strains. ENU sensitivity of the WT strain bearing p5BAD-*dinB* was similar to that of the *mutS*$^\beta$ strain containing the Pol IV-expressing plasmid. About 42 and 54% of WT and *mutS*$^\beta$ cells harboring p5BAD-*dinB* survived after the treatment with ENU, respectively. Conversely, ENU sensitivity of the WT and *mutS*$^\beta$ strains bearing p5BAD was comparable to that of empty cells. Exposure to this alkylating agent did not induce the SOS response as it was tested using the luminescent reporter of the *lexA* promoter transcription (Supplementary Figure S10). The WT, *mutS*$^\beta$, *dinB* and *mutS*$^\beta$ *dinB* strains showed similar luminescence levels to a SOS-deficient PAO1 strain expressing the uncleavable LexAG86V version (36) in plates containing ENU. These findings suggest that disruption of the MutS–β clamp interaction does not appear to affect the ability of Pol IV to bypass ENU-induced lesions. This uncontrolled action of Pol IV by MutS may be owing to that Pol IV replication of ENU lesions is error-free, and not to induction of the SOS response by the alkylating agent.

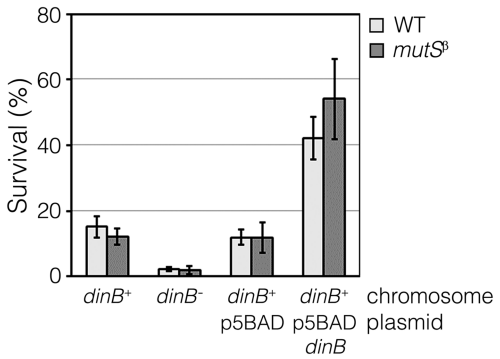

Figure 7. Analysis of sensitivity to ENU-induced DNA lesions. Sensitivity to ENU was measured in cells containing the chromosomal *dinB* allele (*dinB*+, WT and *mutS*$^\beta$ strains) or carrying a deletion of *dinB* (*dinB*-, *dinB* and *mutS*$^\beta$*dinB* strains), and WT and *mutS*$^\beta$ cells containing the empty p5BAD or p5BAD-*dinB*. Data represent mean ± standard deviation ($n = 3$).

MutS and Pol IV expression levels in exponentially growing cells

MutS and MutS$^\beta$ levels were measured by western blot assays in WT and *mutS*$^\beta$ cells, respectively, carrying a C-terminal fusion of the chromosomal encoded MutS or MutS$^\beta$ to a *Strep* II tag (Supplementary Figure S11). Cellular levels of MutS were indistinguishable from that of MutS$^\beta$ in exponentially growing cells expressing Pol IV basal levels, as well as when Pol IV and Pol IV-Δ5 were overexpressed from p5BAD after induction with 0.050% arabinose. These assays confirm that MutS and MutS$^\beta$ expression are similar and thus, the major impact of Pol IV on mutagenesis in the *mutS*$^\beta$ background is not due to differences between MutS and MutS$^\beta$ levels. Likewise, Pol IV expres-

sion levels were tested in WT and *mutS*$^{\beta}$ cells carrying an N-terminal fusion of the chromosomal encoded Pol IV to a His_6 tag. In both strains, Pol IV was not detected by western blotting using polyclonal or monoclonal antibodies raised against the His_6 tag (data not shown). This is in agreement with a previous work (5), where a polyclonal anti-Pol IV serum was used for detecting Pol IV. Thus, Pol IV shows a low undetectable expression level in *P. aeruginosa* cells.

DISCUSSION

According to our biochemical data, *P. aeruginosa* Pol IV and MutS interacted with β clamp. The contact between the hydrophobic cleft of β clamp and the conserved binding motif of Pol IV and MutS was the main anchoring interaction as indicated by the impaired interaction of the Pol IV-Δ5 and MutS$^{\beta}$ mutant versions. Confirming the importance of this contact, we showed that a peptide spanning the CBM of MutS inhibited Pol IV–β clamp and MutS–β clamp interactions. Notably, MutS was able to prevent Pol IV binding to β clamp, as well as to disrupt the Pol IV–β clamp complex. These findings may be explained by the facts that both MutS and Pol IV competed for binding to the protein-binding cleft of the ring, and the MutS–β clamp interaction exhibited a higher affinity than the Pol IV–β clamp interaction. In our experiments we did not detect the formation of a ternary complex consisting of Pol IV, MutS and β clamp as it has been reported for other interacting partners (26,29,50–51). It is likely that MutS binding to β clamp impedes the simultaneous association of Pol IV by occupying both clefts, since MutS has four CBMs per molecule, or by sterically inhibiting access of Pol IV to the ring. In addition, we observed that the affinity of the MutS–β clamp interaction increased in the presence of a GT primed-DNA, which may be important for targeting MutS to β clamp molecules that are bound to a primed site and close to a mismatch (see below the implications of this assumption in the context of our proposed mechanism). It is possible that a conformational change in MutS and β clamp brought about by binding to this DNA structure may have promoted the interaction between these factors. The crystal structures of β clamp in complex with a primed-DNA and MutS bound to mismatched bases differ from those in the apo form, suggesting that binding to these DNA structures induces a conformational change in both proteins (33,52). Regulation of the association between binding partners and β clamp by DNA structures has been documented. For example, the Pol III core (a complex formed by the α polymerase, ϵ exonuclease and θ subunits) develops a higher affinity for β clamp when the ring is placed on primed-DNA, which assures a correct assembly of the replicative Pol at a primed site to initiate processive synthesis (42). We noticed striking effects of DNA structures on the ability of MutS to regulate the Pol IV interaction with β clamp. MutS exerted a more strict control over Pol IV binding to β clamp in the presence of a primed-DNA containing or not a mismatch, may be ensuring that the Pol IV control function of MutS is restricted to replication sites. In contrast, a single-stranded DNA substrate abolished the capability of MutS for limiting the Pol IV–β clamp interaction. Single-stranded DNA regions accumulate after DNA damage, which triggers the SOS response by activating the co-protease activity of RecA that facilitates autocleavage of LexA (48). Thus, it is possible that MutS does not control Pol IV under these stressful conditions wherein the mutagenic activity of Pol IV is necessary to generate adaptive variants.

Our data also demonstrate that basal levels of Pol IV contribute to the normal chromosomal error rate in *P. aeruginosa* cells when MutS does not interact with the β clamp processivity factor. The following lines of evidence support this conclusion: (i) Pol IV deficiency significantly decreased AT > CG, CG > AT and AT > GC base substitution rates in the *mutS*$^{\beta}$ strain. In contrast, and similarly to previous studies in *E. coli* (10), the mutation spectrum was not modified by inactivation of the chromosomal *dinB* gene in the WT strain: (ii) the *mutS*$^{\beta}$ strain showed a Pol IV-dependent strand bias of mutation, which was not detected in the WT strain: (iii) a higher proportion of base substitutions was present at sites involving the Pol IV-preferred sequence G**X**C context in the *mutS*$^{\beta}$ strain compared to the WT strain. At higher Pol IV levels, obtained by ectopic expression, this alternative Pol increased mutations on the *P. aeruginosa* chromosome in a dose-dependent manner in the *mutS*$^{\beta}$ strain, whereas such mutagenesis was limited in the WT strain. This effect was not observed with the Pol IV-Δ5 mutant, demonstrating the functional importance of the association with the processivity factor to target Pol IV to its DNA substrate. On the other hand, the ability of Pol IV to promote survival by bypassing ENU-induced DNA lesions did not differ between the WT and *mutS*$^{\beta}$ strains containing basal or higher Pol IV expression levels. Thus, the MutS–β clamp association limits Pol IV mutagenesis resulting from the low fidelity replication of undamaged DNA, but it does not affect accurate DNA synthesis by Pol IV as in TLS of alkylating lesions.

In vitro studies have shown that Pol IV takes control of the primer terminus from a stalled or, much less efficiently, an actively replicating Pol III HE (comprised of Pol III core, DnaX clamp loader and β clamp) (26–27,29). Moreover, Pol IV is also able to recruit β clamp from Pol III HE replisomes, where the replicative Pol acts in concert with the DnaB helicase and the DnaG primase (25,28). At Pol IV/Pol III HE ratios similar to that found in exponentially growing *E. coli* cells, the Pol switch proceeds through an intermediate in which Pol III HE and Pol IV bind to the same β clamp without dissociation of the replicative Pol from the ring (26,29). Pol III HE contains a high affinity CBM in the α catalytic subunit and a weak CBM in the ϵ exonuclease subunit (50,51). Both sites in the β dimer are occupied simultaneously by the CBMs of Pol III HE during processive replication, whereas breakage of the ϵ–β clamp interaction may occur during proofreading to transfer the primer template from α to ϵ (50,51). It has been suggested that Pol IV is bound to β clamp in an inactive state by contacting a secondary site and that it can switch with Pol III HE by gaining access to the cleft that is weakly bound by ϵ (26–27,50). Pol III HE can remain bound to the ring by contacting the opposing cleft of β clamp with the CBM of α in an inactive conformation while Pol IV is carrying out synthesis (26). These data support a model in which Pol IV is able to associate with β clamp at the replisome without interfering with Pol III HE replication during normal growth, thereby al-

lowing a rapid exchange when Pol III HE is stalled (27,29). However, the biological significance of this model remains largely untested. It is not clear whether Pol IV is constitutively a part of the replisome. A recent work reported that fluorescently tagged Pol IV localizes to the nucleoid in *E. coli* cells under normal growth conditions (53). This localization differs from that of replisome components, which form discrete foci (54), suggesting that this Pol may not be associated with the replicative machinery, despite its high basal concentration. Similarly, *P. aeruginosa* Pol IV may not be associated with β clamp resident in the replisome, since it exhibited a low, undetectable expression level and a weak affinity for the ring. Thus, Pol IV may be recruited to the replication fork for switching with Pol III HE.

Within the framework of the present manuscript, and in accordance with previously published findings, we propose the following mechanism for the control of Pol IV–β clamp interaction by MutS (Figure 8). Diagram A: Pol III HE has control of the primed-DNA during normal replication by occupying both clefts in the polymerization mode. Diagram B: transient stalling of the replication machinery, owing to inability to extend a terminal mismatch generated by Pol III HE or encountering a lesion, can signal the recruitment of Pol IV to the replisome. Pol IV gains access to the primer terminus from Pol III HE by taking the cleft from ϵ while Pol III HE remains bound to the second cleft by the CBM of α. Diagram C: if Pol IV activity generates a mismatch, by introducing an incorrect nucleotide or extending a terminal mismatch, MutS is able to be recruited since its affinity for β clamp increases in the presence of the mispair. Thus, MutS competes with Pol IV on β clamp, leading to its dissociation from the cleft. Diagram D: this could provide the opportunity for the ϵ exonuclease to access the primer terminus, resulting in the excision of the incorrect nucleotide. After error removal, MutS loses affinity for β clamp and leaves the cleft on the ring. Pol III HE ultimately regains control of the primed-site and resumes processive DNA synthesis. Our model predicts that Pol IV can effectively act to alleviate stalling of the replication fork, but if its activity generates a mismatch, MutS can limit further replication by Pol IV and impede the fixation of mutations. Alternatively, MutS could also control the action of Pol IV associated with β clamps that are not directly located at the replisome. It has been shown that β clamp is not immediately unloaded between successive Okazaki fragments but, remains bound to the newly synthesized DNA for a prolonged period of time, resulting in the accumulation of β clamp molecules behind the fork (54,55). If MutS regulates binding of Pol IV to these β clamp molecules, the exonuclease activity of Pol II could be involved in the removal of mispairs generated by Pol IV. Consistent with their role in surveying the replication products of Pol IV, the ϵ exonuclease and Pol II affect several Pol IV-dependent mutagenic phenomena (9,56). In addition, it has been demonstrated that the eukaryotic heterodimer Msh2/Msh6 suppress UV-induced mutagenesis by mediating the excision of incorrect nucleotides introduced by error-prone TLS Pols (57).

Of relevance to our model, we estimated that the concentration of MutS in exponentially growing *P. aeruginosa* cells is ∼265 nM, a lower value than the K_D (∼1 μM) of the MutS–β clamp interaction. Since MutS binding to β clamp significantly increased (∼3.5-fold) in the presence of a primed-DNA containing a mispair, one could assume that MutS only interacts with the processivity factor when a mismatch is generated during replication. Cytological studies in exponentially growing bacteria have shown that MutS must eventually contact β clamp resident in the replisome. MutS fused to a fluorescent protein colocalizes with the chromosome in all cells, but it forms foci in a subset of cells (18,58). These foci are often coincident with foci associated with replisome components, and their formation depends on the MutS interaction with β clamp (18). Accordingly, when a fusion of the 57 C-terminal residues containing the CBM from *P. aeruginosa* MutS to the Maltose Binding Protein was affinity purified from PAO1 extracts, β clamp and the α catalytic subunit of Pol III HE co-eluted with the fusion as identified by LC-MS/MS (data not shown). As we did not detect interaction between MutS and α *in vitro* (Supplementary Figure S4C), these data indicate that MutS may form a complex with β clamp and the α subunit of Pol III.

How does MutS separate its function in the regulation of Pol IV access to replication sites from that in the conserved DNA MMR pathway? MutS is best known in the context of MMR, removing bases mis-inserted during DNA replication. The repair process is initiated when MutS recognizes and binds to mismatches. Subsequently, mismatch-bound MutS promotes loading of the other key MMR protein, MutL, onto DNA activating downstream steps that include discrimination of newly synthesized strand, excision of the erroneous base and strand re-synthesis (59). MutL coordinates MMR processes by interacting with MutS and the majority of the proteins implicated in the subsequent stages. Critical for correct MMR functioning is the interaction between MutS and MutL, which links mismatch recognition with the downstream events. We found that MutS interaction with MutL was inhibited when it formed a complex with β clamp (Supplementary Figure S12A). In contrast, the $MutS^{\beta}$ mutant version interacted with MutL to the same extent in the presence of β clamp (Supplementary Figure S12B). Based on these data, we hypothesize that the interaction of MutS with β clamp favors its action in the regulation of Pol IV access to replication sites and limits MutS activity in repair processes. Thus, when mismatches generated by Pol IV activity are close to β clamp, they can be detected by MutS and removed according to our proposed mechanism. These mispairs are not subjected to MMR since the interaction of MutS with β clamp inhibits its association with MutL. When mismatches generated by Pol IV (or replicative Pols) are far away from β clamp, MutS can interact with MutL and initiate the MMR pathway.

MutS from other bacteria also interacts with the processivity factor (13,18). This interaction plays a role in the MMR of *Bacillus subtilis* by recruiting MutS to sites of DNA replication and allowing for efficient mismatch detection (18,60), but not in the MMR of *E. coli* and *P. aeruginosa* (19,35), which raises questions about their functional significance in both organisms. Our findings shed light on the role of this interaction in *P. aeruginosa* and it is expected that the features described here can be extrapolated to other bacteria. As in the case of gram-negative bacteria, a limited contribution of the MutSα-PCNA association with the MutSα-dependent MMR pathway has been re-

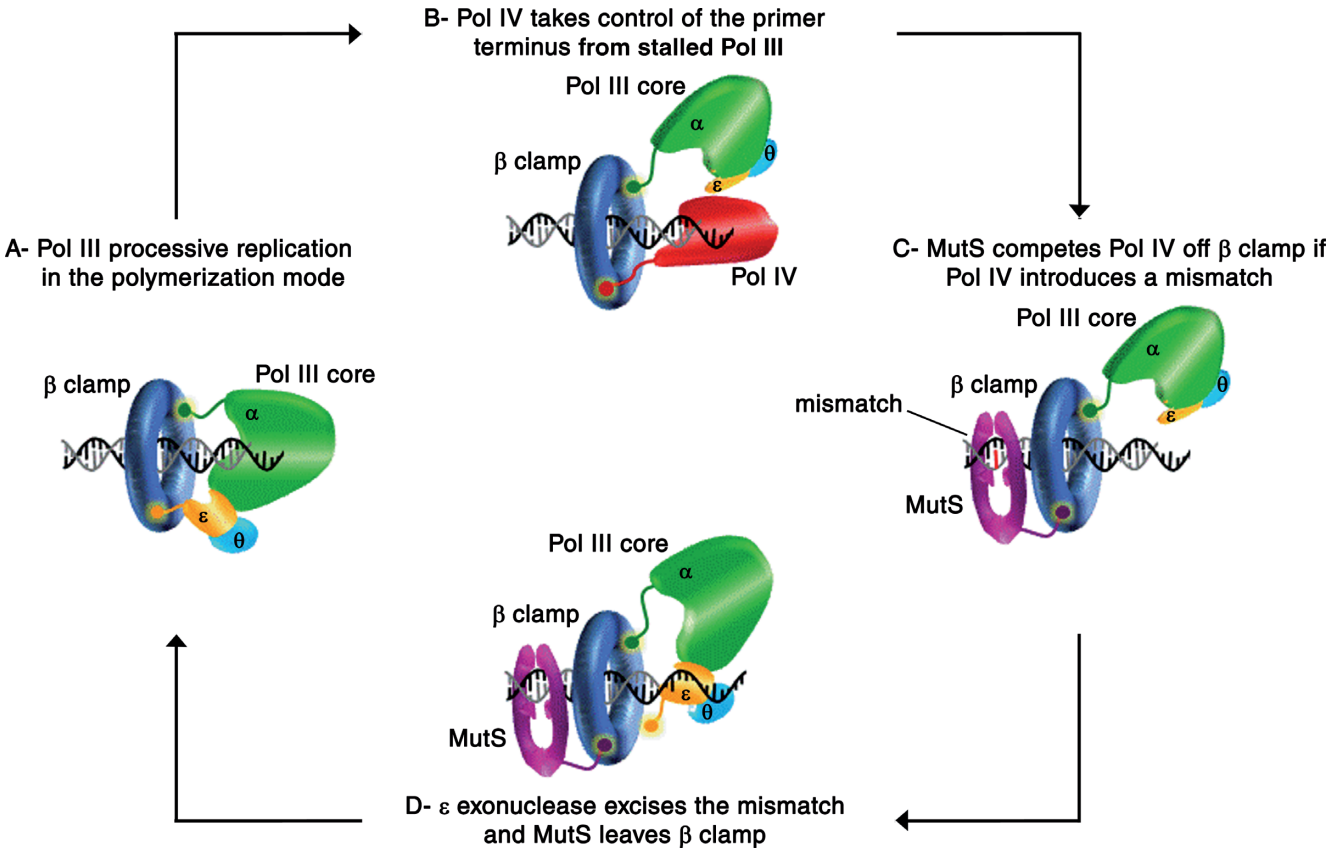

Figure 8. Proposed model for the control of Pol IV–β clamp interaction by MutS. Our model illustrates the action on a single primer terminus, and it does not depict other replisome components. Each of the CBMs is shown as balls. This model was proposed based on previous studies of the Pol III HE/Pol IV switching (**A** and **B**), our findings (**C**) and a postulated action of the ε exonuclease in the excision of mismatches generated by Pol IV (**D**). See 'Discussion' section for details regarding this model.

ported in eukaryotes (20,21). Importantly, MutSα regulates Pol κ- and Pol η-dependent TLS in human cells by promoting monoubiquitination of PCNA in response to UV and oxidative damage, respectively (61,62). This transient post-translational modification of PCNA facilitates recruitment of these TLS Pols and their exchange with the replicative Pol for lesion bypass. Hence, MutS may modulate the action of alternative Pols in both bacteria and eukaryotes.

A high proportion of MutS-deficient cells of *P. aeruginosa* and other pathogenic bacteria often arise during long-term infections (63). Genome sequencing of *P. aeruginosa* isolates taken from chronically infected patients with cystic fibrosis revealed that the homopolymeric tracts of identical nucleotides are preferentially mutated in hypermutators carrying an inactive *mutS* allele (64,65). These homopolymeric tracks are more common in genes related with the cell envelope composition, suggesting that these mutations have been selected to evade the host immune response. Thus, this differential mutagenesis associated with the hypermutator phenotype may facilitate the establishment of long-term existence in the stressful conditions imposed by the human host environment. The presence of homopolymeric tracks in specific genes and the modulation of their mutation rates through inactivation of *mutS* have been shown to be important for pathogenesis and host adaptation of several pathogens (66). Importantly, Pol IV strongly promotes single nucleotide deletions within homopolymeric tracks (5,11,43,67). It is tempting to speculate that Pol IV may be involved in the mutagenesis of genes containing homopolymeric tracks in MutS-deficient cells, where Pol IV is not under the control of MutS, contributing to the pathoadaptation of pathogenic bacteria. Additionally, Pol IV-promoted mutagenesis induced by sublethal levels of different antibiotics depends on the downregulation of MutS (12). Thus, Pol IV mutagenic action under stressful conditions may be determined by the inactivation or depletion of MutS, which is consistent with our proposed model. Accordingly, we found that Pol IV-dependent mutagenesis is not limited by the MutS–β clamp interaction in *P. aeruginosa* cells expressing constitutively the SOS response to stress-induced DNA damage. This may be explained by two ways: (i) MutS expression is downregulated and (ii) the MutS action is regulated by other factors induced as part of the SOS response. Additional studies will be necessary to address these possibilities.

In conclusion, our results reveal important insights into a likely non-canonical function of the MMR protein MutS in the regulation of a replication activity in bacteria involving complex protein–protein and protein–DNA interactions. These findings open up new interrogatives regarding the context and finer details of this mechanism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

Consejo Nacional de Investigaciones Científicas y Técnicas [PIP 20130100360]; Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba [30820110100360CB]; Agencia Nacional de Promoción Científica y Técnica [PICT 2011-1797]. Funding for open access charge: Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 20130100360).
Conflict of interest statement. None declared.

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