Identification of $\beta$ Clamp-DNA Interaction Regions That Impair the Ability of E. coli to Tolerate Specific Classes of DNA Damage

Michael T. Nanfara¹, Vignesh M. P. Babu¹*, Mohamed A. Ghazy¹,², Mark D. Sutton¹*

1 Department of Biochemistry, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY, 14214, United States of America, 2 Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, 11566, Egypt

¤ Current address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, 02139, United States of America

* mdsutton@buffalo.edu

Abstract

The E. coli dnaN-encoded $\beta$ sliding clamp protein plays a pivotal role in managing the actions on DNA of the 5 bacterial DNA polymerases, proteins involved in mismatch repair, as well as several additional proteins involved in DNA replication. Results of in vitro experiments indicate that the loading of $\beta$ clamp onto DNA relies on both the DnaX clamp loader complex as well as several discrete sliding clamp-DNA interactions. However, the importance of these DNA interactions to E. coli viability, as well as the ability of the $\beta$ clamp to support the actions of its numerous partner proteins, have not yet been examined. To determine the contribution of $\beta$ clamp-DNA interactions to the ability of E. coli to cope with different classes of DNA damage, we used alanine scanning to mutate 22 separate residues mapping to 3 distinct $\beta$ clamp surfaces known or nearby those known to contact the DNA template, including residues P20-L27 (referred to here as loop I), H148-Y154 (loop II) and 7 different residues lining the central pore of the $\beta$ clamp through which the DNA template threads. Twenty of these 22 dnaN mutants supported bacterial growth. While none of these 20 conferred sensitivity to hydrogen peroxide or ultra violet light, 12 were sensitized to NFZ, 5 were sensitized to MMS, 8 displayed modestly altered frequencies of DNA damage-induced mutagenesis, and 2 may be impaired for supporting hda function. Taken together, these results demonstrate that discrete $\beta$ clamp-DNA interaction regions contribute to the ability of E. coli to tolerate specific classes of DNA damage.

Introduction

The DnaN/$\beta$ family of sliding clamp proteins plays important roles in coordinating the actions on DNA of a diverse set of proteins involved in DNA replication, repair and translesion synthesis (TLS) (reviewed in [1–3]). To fulfill this role, the clamp must topologically encircle DNA. Clamps are assembled onto DNA by multimeric ATPases called clamp loaders (reviewed in
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The E. coli DnaX clamp loader complex (comprised of τγδψ0ψχ) pries open the dnaN-encoded homodimeric β clamp at one of its two interfaces in an ATP-dependent manner [6–9]. DnaX-ATP, in complex with the open β clamp binds to double strand (ds)/single strand (ss) DNA junctions specifically recognizing the 3'-OH end of the ssDNA [10]. The DnaX clamp loader hydrolyzes bound ATP effecting release of both the clamp and DNA, allowing the β homodimer to close around dsDNA [11]. Once “loaded” onto dsDNA, the β clamp slides freely and recruits its various partner proteins, including each of the five E. coli DNA polymerases (Pols I-V) [12–26]. However, the mechanisms underlying the ability of β clamp to coordinately regulate the actions on DNA of its different partner proteins remain poorly understood (reviewed in [1]).

In addition to DnaX clamp loader, β clamp-DNA interactions also contribute to the loading mechanism [5,27,28]. At least 4 distinct surfaces of the β clamp physically contact the DNA template in a nucleotide sequence-independent manner, including specific residues located within two distinct loops, referred to in this study as loop I (residues P20-L27) and loop II (H148–Y154), a hydrophobic cleft that is known to interact with most if not all of the clamp partner proteins via a conserved clamp binding motif [29], and several residues lining the central pore of the clamp. Position R24 in loop I, together with Q149, Y153 and Y154 in loop II, which contact DNA, contribute to loading of β clamp onto DNA by the DnaX complex in vitro: contributions to loading of other β clamp residues known to contact the DNA template were not investigated [27]. Once loaded, however, these residues were dispensable for Pol III replicase function in vitro [27,28]. A mutant β clamp bearing alanine substitutions of H148–R152 (β148–152) was unable to support E. coli growth, consistent with loop II-DNA interactions serving an essential role in one or more β clamp functions [28]. However, Pol IV (as well as Pol II) interacts with loop II of the β clamp, and this interaction is required for processive replication in vitro [28]. Moreover, physiological levels of β148–152 expressed from a low-copy-number plasmid complemented the temperature sensitive growth phenotype of the dnaN159(Ts) strain provided the lesion bypass dinB-encoded Pol IV was inactivated, suggesting loop II in β clamp contributes to proper coordination of replication and TLS [28,30]. Thus, the contribution of β clamp-DNA interactions to TLS is currently unclear.

In addition to managing the actions of the 5 E. coli Pols to coordinate high fidelity replication with potentially mutagenic TLS, the β clamp also participates in at least 3 additional cellular functions. First, interaction of the β clamp with MutS and MutL contributes to mismatch repair in vivo, ensuring that DNA is replicated faithfully [31,32]. Second, CrfC, a dynamin homolog, exploits its ability to interact with multiple β clamps that accumulate on lagging strand following DNA replication to appropriately position sister replication forks for subsequent chromosome partitioning [33]. Finally, the β clamp interacts with Hda to regulate its ability to dramatically stimulate the ATPase activity of the DnaA replication initiator protein via a process termed regulatory inactivation of DnaA, or RIDA [34]. While the ATP-bound form of DnaA (DnaA-ATP) is active for initiation of DNA replication, the ADP-bound form (DnaA-ADP) is not. Thus, Hda, together with the β clamp, act to regulate initiation by controlling the level of active DnaA in the cell.

The goal of this work was to determine whether residues in the β sliding clamp that directly contact the DNA template contribute to E. coli viability and/or its ability to effectively cope with DNA damage by TLS. To this end, we characterized a total of 22 dnaN mutations targeting positions in loop I, loop II and the central pore of the β clamp. Ten of these 22 mutations specifically targeted residues known to contact the DNA template (Fig 1). The other 12 mutations targeted adjacent residues that did not contact the DNA template in the β clamp-DNA complex, but might in solution. Using a shuffle assay that exploits a ΔdnaN::(kan, sacB) allele, we analyzed phenotypes of these mutant β clamps when expressed at physiological levels from
a low-copy-number plasmid. Twenty of the 22 dnaN mutations supported E. coli viability. Although loop I, loop II and the central pore were each vitally important for protecting E. coli against killing by nitrofurazone (NFZ), only loop I and the central pore were required for protection from methyl methanesulfonate (MMS). In contrast, these three regions of the β clamp were not required for protection from killing by ultraviolet light (UV) or hydrogen peroxide (H₂O₂). Finally, 8 dnaN mutants displayed modestly altered frequencies of DNA damage induced mutagenesis, while 2 others appear to be impaired for supporting hda function. Taken together, these results demonstrate that discrete β clamp-DNA interaction regions contribute to the ability of E. coli to tolerate specific classes of DNA damage.

Materials and Methods

Bacteriological techniques

Bacterial strains and plasmid DNAs used in this study are described in S1 Table; oligonucleotides are described in S2 Table. Strains were constructed using λ recombineering [35], generalized P1vir transduction [36], and CaCl₂-mediated transformation [16], as described in the indicated references. Strains were grown in Luria-Bertani (LB: 10 g/l Difco tryptone, 5 g/l Difco yeast extract, 10 g/l NaCl), or M9 minimal medium (M9: 12.9 g/l Na₂HPO₄•7H₂O, 3 g/l KH₂PO₄, 0.5 g/l NaCl, 1 g/l NH₄Cl) supplemented with 0.1 mM CaCl₂, 2 mM MgCl₂, 40 μg/ml thiamine and 0.5% maltose, as indicated. When appropriate, growth medium was supplemented either with 15 g/l (agar plates) or 7.5 g/l (top agar) Difco agar, and/or 150 μg/ml
ampicillin (Amp), 20 μg/ml kanamycin (Kan), 20 μg/ml chloramphenicol (Cam) and/or 50 μg/ml rifampicin (Rif).

Construction of the ΔdnaN::(kan, sacB) strain

The genetic structure of the ΔdnaN::(kan, sacB) allele, summarized in S1 Fig, was constructed as follows: primers DnaA-BamHI-Fwd and DnaA-NheI-Rev, KanR-NheI-Fwd and KanR-XhoI-Rev, SacB-XhoI-Fwd and SacB-NotI-Rev, and RecF-NotI-Fwd and RecF-XbaI-Rev (S2 Table) were used to PCR amplify fragments corresponding to the 3'-most 762 bp of dnaA, the 945 bp kan gene, the 1,714 bp sacB allele, and the 5'-most 700 bp of recF. These fragments were individually cloned into the Zero Blunt TOPO vector (ThermoFisher) then sequentially joined at the unique NheI (dnaA–kan), XhoI (kan–sacB) and NotI (sacB–recF) sites by standard ligation to Zero Blunt TOPO, ultimately generating plasmid pAKSF-PCR TOPO containing the dnaN::(kan, sacB) locus flanked by dnaA and recF sequences for recombination with the chromosomal dnaN locus. The 4.1 kbp ‘dnaA–ΔdnaN::(kan, sacB)–recF’ fragment was PCR amplified from plasmid pAKSF-PCR TOPO using primers DnaA-BamHI-Fwd and RecF-XbaI-Rev (S2 Table), gel purified, and electroporated into strain MO90-3 as described [28,37], resulting in strain MO90-3-AKSF (S1 Table). Representative transductants, selected on M9 supplemented with maltose and Kan, were confirmed to contain the ΔdnaN::(kan, sacB) allele in place of the normal dnaN+ locus using diagnostic PCR as described [28,37]; growth of these clones was dependent on the lamB::(His6-dnaN+–cam) allele. Representative MO90-3-AKSF clones were further confirmed by nucleotide sequence analysis (Roswell Park Biopolymer Facility, Buffalo, NY) of the PCR amplified ΔdnaN::(kan, sacB) allele from genomic DNA using primers DnaAP and RecF-Bottom.

Cloning mutant dnaN plasmids

Plasmids expressing mutant dnaN alleles were constructed using the QuickChange kit (ThermoFisher Scientific). Oligonucleotides used to generate each mutant are described in S2 Table. Each mutant dnaN allele was sequenced completely (Roswell Park Biopolymer Facility, Buffalo, NY) using primers Beta For1 SP and Beta Rev1 SP.

Measure of the ability of plasmid-expressed dnaN alleles to support viability

The ability of each plasmid-expressed dnaN mutant to support E. coli growth was measured using a shuffle assay. E. coli strain MG1655 was transformed with the indicated β clamp-expressing plasmid. Two independent transformants corresponding to each dnaN mutant were transduced to KanR using P1vir grown on strain MO90-3-AKSF (relevant genotype: ΔdnaN::(kan, sacB)). As a control, select strains were also transduced with P1vir grown on strain MN000 (relevant genotype: tnaA300::Tn10kan dnaN+). Transduction frequency was calculated by dividing the number of KanR CFU by the number of P1vir PFU used in the transduction.

Measurement of steady state levels of mutant β clamp proteins

Western blotting was performed essentially as described previously [30]. Briefly, overnight cultures of the indicated strains were sub-cultured to mid-exponential phase, and 5 x 10^8 cells of each strain were collected by centrifugation. Cell pellets were resuspended in 100 μl 2X SDS-PAGE loading buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 0.1% bromophenyl blue, 20% glycerol, 5% mercaptoethanol). Ten-μl of each whole cell lysate was electrophoresed in 12% SDS-PAGE then transferred to polyvinylidene difluoride (PVDF) using a Trans Blot Turbo...
After transfer, PVDF membranes were washed with TS Buffer (20 mM Tris-HCl (pH 7.6) and 150 mM NaCl) then blocked with TS Buffer containing 0.1% Tween 20 and 2% non-fat dry milk. Washed membranes were probed with rabbit polyclonal anti-β clamp (1:20,000 dilution) antibodies [30]. After washing, PVDF membranes were probed with goat anti-rabbit antibody (1:50,000) and β clamp was detected using the Clarity Western ECL Chemiluminescence substrate (Bio-Rad). Quantitation was performed using a ChemiDoc Imager equipped with the Image Lab software (Bio-Rad). Serial dilutions of wild type extracts were used to verify that observed levels of β clamp were within the linear range of detection.

Measurement of mutant dnaN strain growth rates

Overnight cultures were sub-cultured 1:200 into 200 μl of LB in quadruplicate in sterile 96 well microtiter plates. Plates were incubated at 37°C with shaking, and growth was monitored by measuring the change in optical density at 600 nm (OD_{600 nm}) every 20 min using a FLUOstar Omega microplate reader (Ingen Technologies). The doubling time for each strain was calculated using the doubling time cell calculator (Roth V. 2006 Doubling Time Computing, available from: http://www.doubling-time.com/compute.php).

Susceptibility of mutant dnaN strains to DNA damaging agents

Sensitivity of wild type and mutant dnaN strains to methyl methanesulfonate (MMS) [37], nitrofurazone (NFZ) [38], ultraviolet light (UV) [23,37], or hydrogen peroxide (H_{2}O_{2}) [39] was measured essentially as described in the indicated references. Briefly, sensitivity to MMS and NFZ was measured by spotting 10 μl aliquots of 10-fold serial dilutions of exponential cultures onto LB agar plates supplemented with the indicated concentrations of drug followed by overnight incubation at 37°C. NFZ was suspended in dimethylformamide (DMF), and for the 0 NFZ control, plates were supplemented with DMF alone. For UV, 10-fold serial dilutions of exponential cultures were spotted onto LB agar plates. Spots were allowed to dry then plates were exposed to UV (254 nm) using a 15-W germicidal bulb (General Electric) prior to overnight incubation at 37°C. Sensitivity to H_{2}O_{2} was measured using a disk diffusion assay [39]. One hundred-μl of overnight culture was mixed with 3 ml of molten LB top agar and overlaid on top of an LB agar plate supplemented with Amp. After the top agar solidified (30 min), a sterile 6 mm circular Whatman filter was placed on top of the plate. Ten-μl of either 3% H_{2}O_{2} (Fisher Scientific) or sterile water (as a negative control) was pipetted onto the filter, and the diameter of the zone of clearance was measured after overnight incubation at 37°C.

Ability of mutant dnaN strains to support DNA damage-induced mutagenesis

MMS-induced mutagenesis was measured by inoculating 5 ml of LB supplemented with 0.5–2.0 mM (for dinB^{+} strains), as indicated, or 1 mM MMS (for ΔdinB strains), with 100 μl of exponential cells (OD_{600 nm} = 0.5). Cultures were grown overnight at 37°C. UV-induced mutagenesis was measured using exponential cells (OD_{600 nm} = 0.5) that were washed and resuspended in 0.8% saline. Five hundred-μl of UV (254 nm) irradiated cells (60 J/m²) were inoculated into 4.5 ml of LB and cultured overnight at 37°C. Appropriate dilutions of both MMS and UV treated cells were plated onto either LB agar plates (cell titer) or LB agar supplemented with 50 μg/ml Rif (mutants), and frequencies of MMS- and UV-induced mutagenesis (Rif^{R}) were calculated as described previously [30].
Measurement of oriC/terC ratios

oriC and terC copy numbers were measured using quantitative PCR (qPCR) as described [40]. Briefly, cultures were grown at 37°C in LB medium to an OD_{600nm} = 0.5–0.8. As a control, we used a freshly constructed Δhda::cat strain that was unable to grow at 30°C, indicating it was unsuppressed [40]. The genomic DNA was extracted from 1 ml of each cell culture using the Sigma™ GenElute™ Bacterial Genomic DNA kit as per the manufacturer’s recommendations. Quantitative PCR was performed using Bio-Rad SsoAdvanced™ Universal SYBR® Green Supermix in Bio-Rad CFX96 Connect Real-Time PCR Detection System with manual Cq threshold of 60. PCR reactions (20 μl) contained 10 μl of 2x SYBR Green Supermix, 1.5 μl of genomic DNA (0.3 ng/μl) and 250 nM of each of the oriC-1 and oriC-2 primers, or terC-1 and terC-2 primers, which amplify 150 bp fragments near the origin or terminus, respectively. PCR reactions were heated 98°C for 3 min, followed by 40 cycles with steps of 98°C, 56°C and 72°C for 30s each. The generation of specific PCR products was confirmed using melting curve analysis.

Statistical analysis

Statistical significance for spontaneous mutation was based on 95% confidence intervals (CI) [41]. For all other results, statistical significance was determined using the Evan Miller t-test tool (http://www.evanmiller.org/ab-testing/t-test.html).

Results

Objectives of this study and motivation for the design of dnaN mutations

E. coli β clamp-DNA interactions contribute to the loading of β clamp onto DNA by the DnaX clamp loader complex in vitro [27,28]. However, a comprehensive analysis of the contributions of the different β clamp–DNA interactions to in vivo clamp function has not yet been described. In this work, we used a novel shuffle assay to measure the respective abilities of mutant β clamp proteins to support E. coli viability and to cope with DNA damage by TLS in vivo. Using the β clamp-DNA crystal structure as a guide [27], we performed alanine scanning mutagenesis of three of the four regions in the β clamp demonstrated to contact the DNA template, including loop I (residues P20-L27), loop II (residues H148-Y154) and the inner lining of the β clamp central pore through which dsDNA threads (Fig 1). We targeted residues in the β clamp previously demonstrated to contact DNA, as well as several residues adjacent to these that may also contact DNA under conditions that differ from those used for crystallization [27]. Based on results of in vitro experiments, single mutants of β clamp targeting residues known to contact DNA were still loaded onto DNA fairly efficiently, while loading of double mutants was severely impaired [27]. Therefore, to limit the possibility that a dnaN mutant phenotype was the result of a failure to load that β clamp protein onto DNA, we analyzed phenotypes of single amino acid substitution mutations. We did not examine the hydrophobic cleft, which interacts with ssDNA, due to the fact that this β clamp surface also interacts with the β clamp-binding motif present in most if not all clamp-binding partners, making this region of the β clamp essential for bacterial viability [29,42–44]. Since loop II also interacts with Pol II and Pol IV [28], we decided to substitute each residue in loop I and loop II individually. We hypothesized that if disruption of a particular β clamp–DNA interaction was responsible for an observed mutant phenotype, then mutations targeting nearby residues not involved in DNA interactions should lack this phenotype. Alternatively, if the phenotype was the result of an altered partner protein interaction, then substitution of nearby residues that based on the crystal structure failed to contact the DNA template should confer the mutant phenotype. In addition to alanine

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scanning, we also substituted with glutamic acid two separate β clamp residues lining the central pore of the clamp. We chose a K-to-E substitution hypothesizing that a charge reversal at either of these positions might alter the way in which the β clamp sits on DNA to confer a more pronounced phenotype than would an alanine substitution. One of these residues (K198) directly contacts DNA in the β clamp–DNA crystal, while the other (K12) failed to make contact, but is immediately adjacent to other residues that did contact the DNA template [27]. Furthermore, residues K12 and K198 map to opposite ends of a basic patch lining the central pore of the clamp. We chose a K-to-E substitution hypothesizing that a charge reversal at either of these positions might alter the way in which the β clamp contacts DNA in the clamp–DNA crystal, while the other (K12) failed to make contact, but is immediately adjacent to other residues that did contact the DNA template [27].

Using this shuffle assay, we demonstrated that 20 of the 22 mutant alleles were capable of supporting bacterial growth (Table 2). We were unable to construct the dnaN-D150A and dnaN-R152A shuffle strains. In contrast, these two mutant strains were transduced to KanR by tnaA300::Tn10kan with an efficiency comparable to the dnaN+ control strain (Table 2), supporting the conclusion that the dnaN-D150A and dnaN-R152A mutants were unable to support E. coli viability. Based on the β clamp–DNA crystal structure, D150 did not bind DNA, while residue R152 did. However, the interaction of R152 with DNA was observed in a symmetry-related clamp molecule [27]. Thus, the functional relevance of this DNA interaction is not yet confirmed.

Although viable, the dnaN-G23A, dnaN-R24A and dnaN-V151A strains were transduced ~10-fold less efficiently with ΔdnaN:(kan, sacB) compared to the dnaN+ control (Table 2), suggesting these mutants were partially deficient in one or more essential β clamp function. Nevertheless, these dnaN mutants were transduced with ΔdnaN:(kan, sacB) more than two orders of magnitude more efficiently than was the pWSK29 control. Moreover, this ~10-fold reduction in efficiency compared to the control likely reflects the partial loss of function associated with these mutants.

Residues D150 and R152 in loop II of the β clamp are required for E. coli viability

Using a dnaN shuffle assay, we asked whether physiological levels of site-specific mutant β clamp proteins expressed from a low-copy-number plasmid supported E. coli growth. For this assay, we first transformed E. coli strain MG1655 with the empty cloning plasmid pWSK29, or pWSK29 derivatives expressing either wild type β clamp (pJD100) or one of the mutant clamp proteins (pMN plasmids; see S1 Table). We then quantitatively measured the ability of each mutant dnaN allele to support bacterial growth after replacing the native chromosomal dnaN+ allele with ΔdnaN::(kan, sacB) from strain MO90-3-AKSF using generalized P1vir transduction. As summarized in Table 2, the wild type-expressing plasmid pJD100 supported efficient replacement of the chromosomal dnaN+ allele with ΔdnaN::(kan, sacB) as measured by transduction efficiency to KanR (3.5±0.5 x 10−4 KanR CFU/PFU). In contrast, the empty control plasmid (pWSK29) failed to yield KanR transductants (<2.0 x 10−7 KanR CFU/PFU). In order to confirm the pWSK29 strain was both susceptible to P1vir infection and capable of recombination, we used a P1vir lysate grown on the tnaA300::Tn10kan strain MO900: the tnaA allele is ~80% linked to dnaN+ [45]. As summarized in Table 2, the pJD100 and pWSK29 strains displayed comparable efficiencies of KanR transduction with tnaA300::Tn10kan, confirming that this shuffle assay accurately measures the ability of mutant dnaN alleles to support E. coli viability, similar to a previously described system [46].

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Although viable, the dnaN-G23A, dnaN-R24A and dnaN-V151A strains were transduced ~10-fold less efficiently with ΔdnaN::(kan, sacB) compared to the dnaN+ control (Table 2), suggesting these mutants were partially deficient in one or more essential β clamp function. Nevertheless, these dnaN mutants were transduced with ΔdnaN::(kan, sacB) more than two orders of magnitude more efficiently than was the pWSK29 control. Moreover, this ~10-fold reduction...
in their respective transduction efficiencies was not attributable to a P1\textsuperscript{vir} transduction defect, since these mutants were transduced to Kan\textsuperscript{R} with \textit{tnaA300::Tn10}kan as efficiently as the \textit{dnaN}\textsuperscript{+} control (Table 2). To rule out the possibility that these \textit{dnaN} alleles acquired intragenic suppressor mutations, we isolated the plasmid from both independent isolates of each mutant and sequenced the \textit{dnaN} allele in its entirety. None contained additional mutations. Thus, we conclude that \textit{dnaN-G23A}, \textit{dnaN-R24A} and \textit{dnaN-V151A} are capable of supporting \textit{E. coli} viability. Position R24 contacts the DNA template, while G23 and V151 are immediately adjacent to residues that contact DNA (\cite{27}; see Fig 1).

Based on quantitative Western blotting, each of the mutant \(\beta\) clamp proteins was expressed at levels comparable to the wild type control (Table 3). We also performed growth curves on the 20 viable shuffle strains to determine whether any of the mutant \textit{dnaN} alleles impaired \textit{E. coli} growth, paying particular attention to the \textit{dnaN-G23A}, \textit{dnaN-R24A} and \textit{dnaN-V151A}
strains (Fig 2). As summarized in Table 4, the dnaN-G23A and dnaN-R24A strains grew slightly faster (≤10%) than the wild type control, while the dnaN-V151A mutant was comparable to the wild type control. Five other mutants, including dnaN-Q16A, dnaN-P20A, dnaN-R80A, dnaN-Y153A and dnaN-R197A also grew slightly faster (≤15%) than wild type, while the rest were comparable to the wild type control. Taken together, findings discussed above demonstrate that with the exception of R152, E. coli viability is independent of the known β clamp-DNA interactions.

Mutations targeting loop II and the central pore of the β clamp modestly influence spontaneous mutagenesis

The β clamp interacts with the mismatch repair proteins MutS and MutL [47,48]. Furthermore, based on results of the cryo-electron microscopy (cryo-EM) structure of Pol III core (Pol IIIαβθ) in complex with the C-terminal domain of the τ subunit of DnaX (τC) and the β clamp assembled on DNA, residues 1035–1045 in the OB fold of Pol IIIα interacted with loop I of the β clamp [49]. Importantly, this interaction, among others, was suggested to stabilize the Pol III core-β clamp complex, facilitating processive DNA replication. In order to determine whether

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**Table 2. Abilities of mutant dnaN alleles to support E. coli viability.**

| Mutant class | Plasmid | dnaN allele | P1vir transduction efficiency (Kan<sup>R</sup> CFU/P1vir PFU) for: <sup>a</sup> |
|--------------|---------|-------------|----------------------------------|
|              |         |             | ΔdnaN::(kan, sacB) | dnaN<sup>Δ</sup> tnaA300::Tn10kan |
| Controls     | pJD100  | dnaN<sup>+</sup> | 3.5 (±0.5) x 10<sup>-4</sup> | 2.3 (±0.5) x 10<sup>-4</sup> |
|              | pWSK29  | none        | < 2.0 x 10<sup>-7</sup> | 2.5 (±0.1) x 10<sup>-4</sup> |
| Loop I       | pMN100  | dnaN-P20A   | 0.7 (±0.4) x 10<sup>-4</sup> | nd |
|              | pMN101  | dnaN-L21A   | 3.8 (±1.3) x 10<sup>-4</sup> | nd |
|              | pMN102  | dnaN-G22A   | 2.3 (±0.2) x 10<sup>-4</sup> | nd |
|              | pMN103<sup>b</sup> | dnaN-G23A | 0.2 (±0.1) x 10<sup>-4</sup> | 3.5 (±1.9) x 10<sup>-4</sup> |
|              | pMN104<sup>b</sup> | dnaN-R24A | 0.2 (±0.1) x 10<sup>-4</sup> | 1.1 (±1.0) x 10<sup>-4</sup> |
|              | pMN105  | dnaN-P25A   | 3.1 (±0.4) x 10<sup>-4</sup> | nd |
|              | pMN106  | dnaN-T26A   | 0.7 (±0.1) x 10<sup>-4</sup> | nd |
|              | pMN107  | dnaN-L27A   | 3.4 (±1.8) x 10<sup>-4</sup> | nd |
| Loop II      | pMN108  | dnaN-H148A  | 2.5 (±0.5) x 10<sup>-4</sup> | nd |
|              | pMN109  | dnaN-Q149A  | 1.6 (±0.9) x 10<sup>-4</sup> | nd |
|              | pMN110  | dnaN-D150A  | < 2.0 x 10<sup>-7</sup> | 3.8 (±1.8) x 10<sup>-4</sup> |
|              | pMN111<sup>b</sup> | dnaN-V151A | 0.2 (±0.1) x 10<sup>-4</sup> | 2.8 (±1.1) x 10<sup>-4</sup> |
|              | pMN112  | dnaN-R152A  | < 2.0 x 10<sup>-7</sup> | 3.5 (±1.4) x 10<sup>-4</sup> |
|              | pMN113  | dnaN-Y153A  | 0.8 (±0.3) x 10<sup>-4</sup> | nd |
|              | pMN114  | dnaN-Y154A  | 2.1 (±0.2) x 10<sup>-4</sup> | nd |
| Central pore | pMN115  | dnaN-K12E   | 1.4 (±0.6) x 10<sup>-4</sup> | nd |
|              | pMN116  | dnaN-Q15A   | 2.3 (±0.1) x 10<sup>-4</sup> | nd |
|              | pMN117  | dnaN-Q16A   | 0.6 (±0.1) x 10<sup>-4</sup> | nd |
|              | pMN118  | dnaN-R73A   | 1.4 (±0.5) x 10<sup>-4</sup> | nd |
|              | pMN119  | dnaN-R80A   | 2.2 (±0.1) x 10<sup>-4</sup> | nd |
|              | pMN120  | dnaN-R197A  | 1.8 (±0.4) x 10<sup>-4</sup> | nd |
|              | pMN121  | dnaN-K198E  | 1.2 (±0.3) x 10<sup>-4</sup> | nd |

<sup>a</sup> Results represent the average of two separate determinations, each using an independent dnaN plasmid transformant, ± the range. nd, not determined.

<sup>b</sup> The sequence of the indicated dnaN allele was confirmed in two independent transductants, confirming the lack of an intragenic suppressor mutation.

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loop I, loop II and/or the central pore of the clamp contribute to replication fidelity, through an effect on either Pol III or MutS/MutL function, we measured the frequency of spontaneous mutation for each mutant dnaN strain using Rif\(\text{R}\). Since MMR corrects most replication errors before they become mutations (reviewed in [50,51]), we measured spontaneous mutation frequencies in both \(\text{mutL}^+\) and \(\Delta\text{mutL}\) genetic backgrounds. In the \(\text{mutL}^+\) background, the frequency for each dnaN mutant was comparable, within error, to the wild type control (Table 5), consistent with loop I, loop II and the central pore of the \(\beta\) clamp being dispensable for MMR. However, in the \(\Delta\text{mutL}\) background, the frequency of spontaneous Rif\(\text{R}\) in the dnaN-Q149A mutant was ~2-fold higher than the wild type control, while the dnaN-K12E and dnaN-K198E mutant strains were ~2-fold lower than wild type. Although small, these ~2-fold changes were statistically significant (see 95% CI in Table 5). Spontaneous mutation frequencies for the other \(\Delta\text{mutL} \ dnaN\) mutant strains were indistinguishable from the \(\text{mutL}^+\) control. Residues Q149 and K198 interact with DNA, while K12 is immediately adjacent to several residues that contact the DNA ([27]; see Fig 1). These findings suggest that \(\beta\) clamp-DNA interactions contribute modestly to the fidelity of Pol III replication, and argue that the proposed Pol III\(\alpha\)-\(\beta\) clamp loop I interaction is either dispensable for high fidelity replication in the absence of DNA damage, or that single alanine amino acids substitutions within loop I fail to perturb the Pol III\(\alpha\)-\(\beta\) clamp loop I interaction.

### Table 3. Steady-state expression levels of mutant \(\beta\) clamp proteins.

| Mutant class | dnaN allele | Relative expression level of \(\beta\) clamp * |
|--------------|-------------|-----------------------------------|
| Control      | dnaN*       | 1.0 (≡1.0)                        |
| Loop I       | dnaN-P20A   | 1.40 (±0.24)                      |
|              | dnaN-L21A   | 1.63 (±0.49)                      |
|              | dnaN-G22A   | 1.25 (±0.27)                      |
|              | dnaN-G23A   | 1.12 (±0.02)                      |
|              | dnaN-R24A   | 1.23 (±0.04)                      |
|              | dnaN-P25A   | 1.36 (±0.18)                      |
|              | dnaN-T26A   | 1.21 (±0.01)                      |
|              | dnaN-L27A   | 1.35 (±0.20)                      |
| Loop II      | dnaN-H148A  | 1.27 (±0.26)                      |
|              | dnaN-Q149A  | 1.12 (±0.32)                      |
|              | dnaN-V151A  | 1.42 (±0.17)                      |
|              | dnaN-Y153A  | 1.55 (±0.07)                      |
|              | dnaN-Y154A  | 1.16 (±0.03)                      |
| Central pore | dnaN-K12E   | 0.94 (±0.03)                      |
|              | dnaN-Q15A   | 0.75 (±0.15)                      |
|              | dnaN-Q16A   | 0.99 (±0.39)                      |
|              | dnaN-R73A   | 1.13 (±0.30)                      |
|              | dnaN-R80A   | 1.03 (±0.15)                      |
|              | dnaN-R197A  | 0.90 (±0.35)                      |
|              | dnaN-K198E  | 1.83 (±0.30)                      |

* Respective levels of wild type and mutant \(\beta\) clamp proteins were determined by quantitative Western blotting. Results are expressed relative to \(\beta\) clamp levels observed in the wild type dnaN* strain, which were set equal to 1.0 (≡1.0), and represent the average of two independent determinations, each involving separate whole cell lysate, ± the range.

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Distinct roles for loop I, loop II and the central pore of the β clamp in coping with DNA damage

To gain insight into whether β clamp-DNA interactions contribute to DNA repair and/or DNA damage tolerance, we measured the sensitivity of each dnaN mutant to nitrofurazone (NFZ), methyl methanesulfonate (MMS), ultraviolet light (UV) and hydrogen peroxide.

Table 4. Growth rates of mutant dnaN strains.

| Mutant class  | dnaN allele | Doubling time (min) a |
|---------------|-------------|----------------------|
| Control       | dnaN+       | 47.5 (±1.5)          |
| Loop I        | dnaN-P20A   | 44.3 (±1.4) b        |
|               | dnaN-L21A   | 47.7 (±1.5)          |
|               | dnaN-G22A   | 47.2 (±2.4)          |
|               | dnaN-G23A   | 44.7 (±0.7) b        |
|               | dnaN-R24A   | 42.7 (±1.2) b        |
|               | dnaN-P25A   | 47.4 (±2.3)          |
|               | dnaN-T26A   | 45.3 (±2.1)          |
|               | dnaN-L27A   | 48.8 (±2.7)          |
| Loop II       | dnaN-H148A  | 45.5 (±1.5)          |
|               | dnaN-Q149A  | 47.3 (±1.0)          |
|               | dnaN-V151A  | 44.9 (±2.4)          |
|               | dnaN-Y153A  | 42.8 (±1.5) b        |
|               | dnaN-Y154A  | 45.6 (±2.3)          |
| Central pore  | dnaN-K12E   | 47.8 (±1.4)          |
|               | dnaN-Q15A   | 42.4 (±3.6)          |
|               | dnaN-Q16A   | 40.7 (±3.6) b        |
|               | dnaN-R73A   | 49.7 (±1.1)          |
|               | dnaN-R80A   | 41.9 (±1.0) b        |
|               | dnaN-R197A  | 45.2 (±1.4) b        |
|               | dnaN-K198E  | 49.5 (±1.8)          |

a Values shown represent the average of four separate determinations ± one standard deviation. Growth rates are slightly slower than typical liquid cultures due to culturing in 96 well microtiter plates.

b These values are significantly different from the wild type control (p < 0.05).
Table 5. Effects of mutant dnaN alleles on spontaneous mutagenesis.

| Mutant class | dnaN allele | Spontaneous mutation frequency: \(^a\) | \(\Delta\text{mutL::cat}\) |
|--------------|-------------|-----------------------------------------|-----------------|
| Control      | dnaN          | 6.07 x 10^{-8} (1.67 x 10^{-8}–1.23 x 10^{-7}) | 9.39 x 10^{-8} (8.41 x 10^{-8}–1.29 x 10^{-7}) |
| Loop I       | dnaN-P20A     | 3.31 x 10^{-8} (1.79 x 10^{-8}–1.11 x 10^{-7}) | 1.50 x 10^{-8} (7.69 x 10^{-9}–2.31 x 10^{-8}) |
|              | dnaN-L21A     | 1.09 x 10^{-8} (8.80 x 10^{-9}–5.26 x 10^{-9}) | 1.54 x 10^{-8} (1.30 x 10^{-8}–1.92 x 10^{-8}) |
|              | dnaN-G22A     | 5.30 x 10^{-8} (1.52 x 10^{-8}–1.20 x 10^{-7}) | 1.26 x 10^{-7} (6.40 x 10^{-8}–2.91 x 10^{-7}) |
|              | dnaN-G23A     | 1.51 x 10^{-8} (8.80 x 10^{-9}–3.55 x 10^{-9}) | 1.16 x 10^{-8} (8.45 x 10^{-9}–1.76 x 10^{-8}) |
|              | dnaN-R24A     | 6.76 x 10^{-8} (2.17 x 10^{-8}–1.43 x 10^{-8}) | 1.08 x 10^{-7} (4.62 x 10^{-8}–1.62 x 10^{-7}) |
|              | dnaN-P25A     | 5.76 x 10^{-8} (2.03 x 10^{-8}–7.35 x 10^{-9}) | 9.53 x 10^{-8} (7.50 x 10^{-9}–1.94 x 10^{-8}) |
|              | dnaN-T26A     | 8.62 x 10^{-8} (1.10 x 10^{-8}–3.02 x 10^{-9}) | 1.37 x 10^{-7} (1.10 x 10^{-8}–1.70 x 10^{-8}) |
|              | dnaN-L27A     | 2.66 x 10^{-8} (1.34 x 10^{-8}–1.04 x 10^{-7}) | 8.49 x 10^{-8} (4.94 x 10^{-9}–1.14 x 10^{-8}) |
| Loop II      | dnaN-H148A    | 5.51 x 10^{-8} (1.33 x 10^{-8}–1.48 x 10^{-7}) | 2.35 x 10^{-8} (1.20 x 10^{-8}–5.60 x 10^{-9}) |
|              | dnaN-Q149A    | 4.00 x 10^{-8} (1.49 x 10^{-8}–1.35 x 10^{-7}) | 1.95 x 10^{-7} (1.50 x 10^{-8}–2.83 x 10^{-8}) \(^b\) |
|              | dnaN-V151A    | 2.72 x 10^{-8} (2.08 x 10^{-8}–5.63 x 10^{-8}) | 9.75 x 10^{-8} (7.63 x 10^{-9}–2.54 x 10^{-8}) |
|              | dnaN-Y153A    | 1.81 x 10^{-8} (1.09 x 10^{-8}–7.35 x 10^{-9}) | 2.05 x 10^{-8} (1.24 x 10^{-8}–3.23 x 10^{-9}) |
|              | dnaN-Y154A    | 2.02 x 10^{-8} (1.16 x 10^{-8}–1.17 x 10^{-7}) | 2.57 x 10^{-8} (1.18 x 10^{-8}–3.95 x 10^{-9}) |
| Central pore | dnaN-K12E     | 5.95 x 10^{-8} (5.00 x 10^{-8}–1.52 x 10^{-7}) | 4.60 x 10^{-8} (2.78 x 10^{-8}–5.21 x 10^{-8}) \(^b\) |
|              | dnaN-Q15A     | 1.46 x 10^{-7} (3.29 x 10^{-8}–3.36 x 10^{-7}) | 1.99 x 10^{-8} (1.09 x 10^{-9}–4.18 x 10^{-9}) |
|              | dnaN-Q16A     | 2.94 x 10^{-8} (1.29 x 10^{-8}–1.43 x 10^{-7}) | 8.12 x 10^{-8} (4.55 x 10^{-9}–1.30 x 10^{-8}) |
|              | dnaN-R73A     | 1.10 x 10^{-7} (4.31 x 10^{-8}–2.60 x 10^{-7}) | 6.58 x 10^{-8} (3.29 x 10^{-8}–8.55 x 10^{-9}) |
|              | dnaN-R80A     | 3.21 x 10^{-8} (1.45 x 10^{-8}–9.19 x 10^{-9}) | 8.88 x 10^{-8} (5.07 x 10^{-9}–1.74 x 10^{-8}) |
|              | dnaN-R197A    | 7.81 x 10^{-8} (2.66 x 10^{-8}–1.39 x 10^{-8}) | 4.83 x 10^{-8} (2.15 x 10^{-8}–1.35 x 10^{-8}) |
|              | dnaN-K198E    | 1.03 x 10^{-7} (3.07 x 10^{-8}–1.46 x 10^{-7}) | 5.45 x 10^{-8} (3.33 x 10^{-8}–7.58 x 10^{-9}) \(^b\) |

\(^a\) The frequency of spontaneous mutation was measured as described [46]. Values represent the median of 9 independent determinations. 95% confidence intervals (CI) are indicated [41].

\(^b\) These values are significantly different from the wild type control based on 95% CI.

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(H\(_2\)O\(_2\)). We chose these agents since repair of the DNA damages that they collectively induce requires the majority of the known DNA repair and damage tolerance functions, including TLS by Pol II, Pol IV and Pol V (reviewed in [50]). To measure sensitivity to NFZ and MMS, we spotted 10 μl aliquots of 10-fold serial dilutions of exponential cultures onto agar plates supplemented with the indicated concentrations of the respective chemical. We began with NFZ. As summarized in Fig 3, more than half of the mutants were sensitized to NFZ, including dnaN-P20A, dnaN-G23A, dnaN-R24A and dnaN-T26A in loop I, dnaN-V151A and dnaN-Y153A in loop II, and dnaN-K12E, dnaN-Q16A, dnaN-R73A, dnaN-R80A, dnaN-R197A and dnaN-K198E lining the central pore. Of these 12, 5 corresponded to positions in the β clamp known to contact DNA, including R24, Y153, R73, R80 and K198 [27]. Furthermore, mutations mapping to loop II (V151A), and in particular the central pore (K12E, R73A, R80A, R197A & K198E), displayed a far greater level of NFZ sensitivity than mutations mapping to other positions in the β clamp, suggesting these regions play a more prominent role in coping with NFZ-induced DNA damage.

Compared to NFZ, fewer mutants were sensitized to MMS, including dnaN-L21A and dnaN-T26A in loop I and dnaN-K12E, dnaN-R80A and dnaN-R197A lining the central pore (Fig 4). Of these, only R80 is known to contact DNA [27]. Interestingly, none of the mutations mapping to loop II conferred sensitivity to MMS. It is noteworthy that with the exception of
DNA-L21A, those sensitized to MMS were also sensitized to NFZ (summarized in Table 1).

Interestingly, dnaN-V151A, dnaN-Y153A and dnaN-Y154A appeared modestly resistant to MMS compared to the wild type control (Fig 4), suggesting these mutant clamps are able to cope more efficiently with MMS-induced DNA damage than wild type β.

To measure UV sensitivity, we spotted 10 μl aliquots of 10-fold serial dilutions of exponential cultures onto agar plates, allowed them to dry then irradiated with the indicated dose of UV. As summarized in S2 Fig, none of the dnaN mutants were sensitized to UV. Likewise, based on results of a disk diffusion assay [39], none of the dnaN mutants conferred sensitivity to H₂O₂ (S3 Fig). In summary, a total of 13 dnaN mutants displayed sensitivity to NFZ and/or MMS, with none conferring sensitivity to UV or H₂O₂.
Loop I, loop II and the central pore of the β clamp make distinct contributions to DNA damage-induced mutagenesis

We next asked whether any of the dnaN mutants were impaired for supporting DNA damage-induced mutagenesis. The NFZ and MMS sensitivity observed for several of the dnaN mutants discussed above suggested a possible Pol IV defect. Most DNA damage-induced mutagenesis in E. coli is dependent on Pol V, including that induced by MMS and UV ([52,53]; reviewed in [50]). Although Pol IV is inefficient at catalyzing bypass of UV-induced DNA lesions [54], it plays a biologically important role in accurate bypass of MMS-induced lesions, including N⁳-methyladenine (N³-mdA) [37,55–57]. Thus, the frequency of MMS-induced mutagenesis is a reflection of the relative activities of Pol IV and Pol V. For example, the frequency of MMS-induced mutagenesis is increased by more than 10-fold in the absence of Pol IV function [37,55]. Since we hypothesized that the NFZ and MMS sensitive dnaN mutants were
potentially impaired for Pol IV function, we measured frequencies of mutagenesis induced by MMS or UV. We first measured frequencies for MMS-induced mutagenesis. A total of 5 dnaN mutants displayed an altered frequency of MMS-induced mutagenesis, including dnaN-G22A and dnaN-R24A mapping to loop I, and dnaN-K12E, dnaN-R80A and dnaN-R197A mapping to the central pore (Fig 5A). Of these 5, only R24 and R80 are known to interact with DNA [27]. Interestingly, none of the mutations in loop II affected the frequency of MMS-induced mutagenesis. The mutations in loop I (G22A & R24A) displayed a ~2- to ~3-fold increase in their respective frequencies of MMS-induced mutagenesis, while the mutations in the central pore were impaired either completely (K12E), or by ~50% relative to the wild type control (R80A & R197A) (Fig 5A). While neither loop I mutant was sensitized to MMS, all 3 central

Fig 5. Ability of mutant dnaN strains to support DNA damage-induced mutagenesis. Frequencies of (A) MMS-induced (2 mM) and (B) UV-induced mutagenesis (60 J/m²) were measured as described in Materials and Methods. Results represent the average of 3 independent determinations using 2 independent clones for each dnaN allele ± one standard deviation. Bars are color coded to indicate mutations analyzed map to loop I (red), loop II (blue) or the central pore (green) of the β clamp. Symbols: *, p < 0.05; **, p < 0.001.

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pore mutants were (Fig 4), which may contribute to their reduced frequency of MMS-induced mutagenesis. To determine whether MMS sensitivity of the dnaN-K12E mutant influenced its ability to support induced mutagenesis, we measured mutation frequency over a range of MMS concentrations (0.5 to 2.0 mM). As summarized in S4 Fig, the dnaN-K12E mutant failed to display induced mutagenesis at all MMS concentrations examined. Finally, as discussed below, the 3 dnaN mutants that were impaired for MMS-induced mutagenesis (dnaN-K12E, dnaN-R80A & danN-R197A) were proficient for UV-induced mutagenesis (Fig 5B), indicating that their MMS defect was not the result of a failure to induce SOS, or to properly manage the actions of Pol V.

We next measured respective frequencies of UV-induced mutagenesis. In contrast to its role following exposure to MMS, mutations targeting loop I failed to significantly affect the frequency of UV-induced mutagenesis. Instead, 2 mutations in loop II (dnaN-Q149A & dnaN-V151A) and one in the central pore (dnaN-R73A) displayed altered frequencies (Fig 5B). The dnaN-Q149A and dnaN-R73A mutants exhibited a ~2-fold higher UV-induced mutation frequency compared to the wild type control, while the frequency of the dnaN-V151A mutant was reduced by ~50% (Fig 5B). Importantly, residues Q149 and R73 are known to interact with the DNA template [27]. Taken together, findings discussed above indicate that substitutions in loop I, loop II and the central pore exert modest, albeit distinct effects on MMS- and UV-induced mutagenesis. Several of these mutations map to β clamp residues that make direct contact with the DNA template, providing support for the view that β clamp-DNA interactions contribute to the efficiency of TLS.

The modestly increased frequency of MMS-induced mutagenesis of the dnaN-G22A and dnaN-R24A mutants is dependent on dinB (Pol IV) function

Since Pol IV function attenuates the magnitude of Pol V-dependent mutagenesis in response to MMS, we hypothesized that the increased frequency of MMS-induced mutagenesis observed for the dnaN-G22A and dnaN-R24A mutants (Fig 5A) was the result of a partially impaired ability of these 2 mutant β clamps to properly manage the actions of Pol IV, leading to more robust levels of Pol V dependent mutagenesis. We likewise questioned whether the reduced frequencies of mutagenesis observed for the dnaN-K12E, dnaN-R80A and/or dnaN-R197A mutant strains (Fig 5B) were related to altered Pol IV function. As a test of these hypotheses, we engineered double mutant strains expressing the mutant dnaN alleles together with ΔdinB, and measured their respective abilities to cope with MMS. We first measured MMS sensitivity of the double mutants using the same spot assay discussed above. Although the dnaN-G22A and dnaN-R24A mutants failed to exhibit MMS sensitivity when assayed in the dinB+ background (Fig 4), they were both sensitized to MMS in the ΔdinB background (Fig 6A). These results suggest that Pol IV contributes to the ability of these mutants to cope with MMS-induced DNA damage. In contrast to dnaN-G22A and dnaN-R24A, the dnaN-K12E, dnaN-R80A and dnaN-R197A mutants exhibited similar levels of MMS sensitivity compared to the wild type control, irrespective of dinB function (compare Figs 4 & 6A), indicating their MMS sensitivity was independent of Pol IV.

We next measured MMS-induced mutagenesis. Whereas the frequency of MMS-induced mutagenesis of the dnaN-G22A mutant was indistinguishable from the wild type control, that of dnaN-R24A was reduced to ~30% of the control (Fig 6B). Thus, the increased frequency of MMS-induced mutagenesis observed for the dnaN-G22A and dnaN-R24A mutants was completely dependent on Pol IV function. In contrast to the loop I mutants, which were to varying degrees epistatic with ΔdinB, the inability of the dnaN-K12E, dnaN-R80A and dnaN-R197A
mutants to support efficient MMS-induced mutagenesis was unrelated to dinB function (compare Figs 5A & 6B). Taken together, these results demonstrate a role for positions G22 and R24 in loop I of the β clamp in ensuring the efficiency of Pol IV TLS in vivo. In contrast, the impaired ability of the central pore mutants bearing substitution of positions K12, R80 and R197 to support MMS-induced mutagenesis is likely the result of their MMS hypersensitivity.

Loop II and the central pore of the β clamp, and possibly loop I contribute to hda function

Several of the dnaN mutants that exhibited sensitivity to NFZ and/or MMS were proficient for DNA-damage induced mutagenesis, indicating that they were impaired for one or more
functions distinct from TLS. Loss of seqA function leads to UV sensitivity due to over initiation of DNA replication [58]. Since Hda interacts with β camp to regulate activity of DnaA in initiation [34], we hypothesized that one or more of the dnaN mutants that were sensitized to NFZ and/or MMS may be impaired for hda function. As a test of this hypothesis, we used qPCR to measure oriC/terC ratios in these strains. If these dnaN mutants were impaired for supporting hda function then we would expect them to possess an elevated oriC copy number relative to terC due to over initiation. As summarized in Fig 7, loss of hda function resulted in an almost 3-fold increase in the oriC/terC ratio, as expected [34,40]. Likewise, the oriC/terC ratio was also elevated ~2-fold and ~3-fold, respectively, in the dnaN-V151A and dnaN-R73A strains. Although failing to reach statistical significance, oriC/terC ratios were also modestly elevated in the dnaN-P20A, dnaN-L21A and dnaN-R24A strains. Residues R24 and R73 interact with DNA, while P20, L21 and V151 failed to do so in the crystal [27]. Taken together, these findings suggest that dnaN-V151A and dnaN-R73A are impaired for regulating initiation, consistent with a role for β clamp-DNA interactions in supporting hda function.

**Discussion**

The goal of this work was to determine whether residues in the β sliding clamp that directly contact the DNA template contribute to E. coli viability or its ability to effectively cope with DNA damage by TLS. We analyzed a total of 22 different dnaN mutations collectively targeting
loop I, loop II and the central pore of the β clamp. Ten of these 22 mutations targeted positions in the β clamp previously demonstrated to contact the DNA template, while the other 12 affected nearby residues [27]. Seventeen of these 22 mutations displayed one or more mutant phenotypes: 2 were unable to support bacterial growth, 3 more were modestly impaired in this regard, 12 were sensitized to NFZ, 5 were sensitized to MMS, 8 displayed modestly altered frequencies of DNA damage-induced mutagenesis and 2 appear to be impaired for supporting hda function (summarized in Table 1 & Fig 8). With the notable exceptions of D150 and R152, both of which failed to support *E. coli* viability, the other dnaN mutations supported normal growth, arguing they retained the ability to be loaded onto DNA in vivo well enough to support chromosomal replication. Taken together, these results demonstrate that discrete β clamp-DNA interaction regions contribute to the ability of *E. coli* to tolerate DNA damage (Fig 8A & 8B). While the individual β clamp-DNA interactions were largely dispensable for DNA damage-induced mutagenesis, they nevertheless contribute significantly to its efficiency in vivo (Fig 8C & 8D). Finally, we acknowledge the possibility that β clamp-DNA interactions contribute more significantly to TLS than suggested here by phenotypes of single amino acid substitutions in the clamp.

Loop II of the β clamp was unique among the 3 surfaces examined in this work in that it was the only region in which mutations (D150A & R152A) failed to support *E. coli* viability (Table 2). Although viable, the dnaN-G23A, dnaN-R24A mutants in loop I, and the dnaN-V151A mutant in loop II were each constructed ~10-fold less efficiently compared to the wild type control (Table 2). We suggest this is due to each of these mutants being impaired for one or more β clamp functions that become essential for viability under specific circumstances not routinely encountered during growth. In contrast to β-R24A, which was modestly impaired for loading onto DNA *in vitro*, the ability of DnaX to load the mutant β-R152A clamp protein was not examined. Thus, it is unclear whether the inability of the dnaN-R152A mutant to support viability is related to a loading defect. Regardless, neither D150 nor V151 of the β clamp interacted with DNA in the β clamp-DNA crystal [27]. Neuwald suggested that positions Q149 and K198 sense DNA within the central pore of the β clamp and relay this signal to residues D150 and R152 as part of the clamp loading mechanism [59]. Thus, the inability of dnaN-D150A and dnaN-R152A to support *E. coli* viability may relate to a loading defect. Alternatively, it is possible that these residues perform one or more roles critical to *E. coli* viability in addition to loading the β clamp onto DNA.

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**Fig 8. Summary of the major phenotypes observed for dnaN mutations.** Residues that played a significant role in protecting *E. coli* against killing by (A) NFZ or (B) MMS, or that when substituted significantly affected (C) the frequency of mutagenesis induced by exposure to UV light, or (D) the ratio of oriC to terC, suggesting an hda defect, are represented on the structure of the β clamp-DNA complex. Residues are color coded by position, with those in loop I colored red, loop II colored blue, and the central pore colored green. This image was generated using the MacPyMol Molecular Graphics System, Ver. 1.7.4 Schrodinger, LLC and the coordinates for the crystal structure of the β clamp-DNA complex (PDB: 3BEP).

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Whereas Pol IV catalyzes accurate bypass of MMS-induced DNA lesions, Pol V is responsible for most mutations resulting from MMS exposure [52,53,55]. We observed a small yet statistically significant ~2- to ~3-fold increase in the frequency of MMS-induced mutagenesis for the dnaN-G22A and dnaN-R24A mutants compared to the wild type dnaN control (Fig 5A). Importantly, this increase was absolutely dependent on dinB (Pol IV) function (Fig 6B). Taken together, these findings suggest that the G22A and R24A substitutions either impair the efficiency with which Pol IV is recruited to sites of MMS-induced DNA damage, or interfere with the ability of the β clamp to properly manage the actions of Pol IV after it is recruited. As a result, Pol V plays a greater role in bypassing MMS-induced lesions, explaining the ~2- to ~3-fold increase in mutation frequency (Fig 5A). Although G22 of the β clamp failed to contact DNA in the crystal [27], flexibility at this position may contribute to the ability of R24 to interact with DNA [60–62]. In this case, the modest Pol IV defect observed for dnaN-G22A and dnaN-R24A could result from impaired β clamp-DNA interactions. Alternatively, Pol IV may contact loop I of the β clamp, and the G22A and R24A mutations may interfere with this interaction. Regardless, it is important to point out that the dnaN-G22A and dnaN-R24A mutants retained a partial ability to manage Pol IV, since their respective frequencies of MMS-induced mutagenesis were still higher than the isogenic ΔdinB strain (~20 x 10^{-7} and ~27 x 10^{-7} for the dnaN mutants in the presence of dinB$, respectively, compared to ~30 x 10^{-6} for the dnaN$\Delta$dinB strain; see Figs 5 & 6). Finally, in addition to their role in Pol IV function, positions G22 and R24 in loop I of the β clamp appear to play an additional role in protecting E. coli against killing by MMS independent of Pol IV function (Fig 6A).

We previously presented evidence that β^{148–152} (bearing alanine substitutions of residues H148-R152) was impaired for Pol V mutagenesis in vivo [28,30]. Consistent with this view, two of the mutations analyzed in this work that mapped to loop II of the β clamp significantly altered the frequency of UV-induced mutagenesis (Fig 5B). The mutation frequency of dnaN-Q149A was elevated ~2-fold compared to the wild type control, while that of dnaN-V151A was reduced ~2-fold. Patoli et al. determined that position N359 of the UmuC subunit of Pol V hydrogen bonds to residue R152 of the β clamp [63]. R152 of the symmetry-related β molecule interacted with DNA [27]. Thus, Pol V and the DNA may compete with each other for binding loop II of the β clamp. The ~2-fold increase in UV-induced mutagenesis observed for the dnaN-Q149A mutant may result from a DNA binding defect, shifting the equilibrium away from β clamp-DNA interaction and towards β clamp-Pol V interaction. Likewise, the ~2-fold reduction in mutation frequency for the dnaN-V151A mutant may reflect the reciprocal situation in which the mutation impairs the β clamp-Pol V interaction. Alternatively, since the β clamp functions as a homodimer, R152 in one β protomer may interact with UmuC, while R152 in the other β protomer may contact the DNA template. In this case, altered Pol V function in vivo may result from effects on β clamp-DNA interactions, as well as secondary effects of these mutations on β clamp-Pol V interactions. Finally, β^{148–152} was impaired for interaction with both Pol II and Pol IV in vitro, and was unable to support processive replication by these Pols in vitro [28]. Although the single substitution mutations used here failed to exert an obvious effect on Pol IV function in vivo, our prior in vivo and in vitro results with β^{148–152} [28], taken together with our in vivo findings presented here, indicate that loop II of the β clamp contributes to function of all 3 E. coli TLS Pols. Taken together, these findings suggest that multiple substitutions within loop II may be required to observe a Pol II and Pol IV defect in vivo. These findings additionally suggest that there are significant differences in how these 3 TLS Pols interact with loop II of the β clamp.

While mutations mapping to loop I and loop II conferred sensitivity to NFZ, sensitivity of the dnaN mutations mapping to the central pore was particularly robust (Fig 3). NFZ-induced lesions are either repaired by nucleotide excision repair (NER) or bypassed accurately by Pol
IV [38,64,65]. All 6 of the NFZ sensitive dnaN mutations mapping to the central pore were proficient for NER, based on their ability to tolerate UV light (S2 Fig). Likewise, 3 (Q16A, R73A & K198A) of the 6 were tolerant of MMS, indicating they were largely proficient for Pol IV function. Thus, the mechanistic basis for the NFZ sensitivity of these dnaN mutations is unclear. Ona et al. demonstrated that exposure of E. coli to high concentrations of NFZ (200 μM) for short periods of time inhibited DNA replication without causing DNA damage [64]. Thus, the dnaN mutants that were sensitized to NFZ may be impaired for interaction with one or more subunits of Pol III, resulting in a less stable replisome that fails to cope efficiently with NFZ. The other 3 dnaN mutations (K12E, R80A & R197A) conferring NFZ sensitivity were significantly sensitized to MMS, and were impaired to varying degrees for MMS-induced mutagenesis (Fig 5A). That these 3 dnaN mutants were proficient in UV-induced mutagenesis (Fig 5B) argues that their inability to support MMS-induced mutagenesis was the result of their MMS hypersensitivity. The mechanistic basis for this MMS sensitivity is currently unknown, but could be attributable to a Pol III and/or Pol I replication defect. Finally, it is possible that the dnaN mutants with increased sensitivity to NFZ and/or MMS are less stable on DNA, or are more rapidly unloaded, possibly due to their impaired ability to interact with one or more partner proteins. This would lead to fewer clamps on DNA, which could reduce the efficiency with which NFZ- and MMS-induced lesions are repaired. Consistent with this model, all 7 of the dnaN mutants that grew more quickly than the wild type strain were sensitive to NFZ, and several were sensitive to MMS. Faster recycling of these mutant β clamp proteins off of the DNA and into the soluble pool used by DnaX to repeatedly load clamp onto lagging strand could also lead to more rapid replication, explaining their increased growth rate (Table 1). Furthermore, if these mutant clamps did in fact support faster replication, then the forks in these mutants might collide more frequently with either unrepaired lesions or ssDNA gaps generated during the repair process. This, in turn, would contribute to increased sensitivity to these DNA damaging agents.

While increased sensitivity to DNA damage is typically thought to be the result of an impaired ability to properly cope with DNA damage using accurate repair or potentially mutagenic TLS, it can also be the result of over initiation of DNA replication. In this case, sensitivity results from more frequent collisions between the closely spaced replication forks and DNA lesions or repair intermediates [58,66]. In order to determine whether any of the 13 dnaN mutants that were sensitized to NFZ and/or MMS were impaired for supporting hda function, we used qPCR to measure their oriC/terC ratios. As summarized in Table 1 and Fig 7E, dnaN-V151A and dnaN-R73A exhibited a significantly increased oriC copy number compared to the dnaN+ control. Although not statistically significant, oriC/terC ratios were also modestly elevated in the dnaN-P20A, dnaN-L21A and dnaN-R24A strains compared to the wild type control, suggesting that loop I of the β clamp play a role in managing the actions of Hda in vivo. While the elevated oriC copy number observed in these strains suggests that these dnaN mutants are impaired for managing the ability of Hda to regulate the activity of DnaA in initiation of DNA replication, we cannot rule out the possibility that this phenotype is due at least in part to these dnaN mutants being impaired for supporting Pol III replication. In this case, fewer forks would reach terC, contributing to an elevated oriC/terC ratio.

Nine of the 12 residues that did not interact with DNA in the β clamp-DNA crystal examined in this work contributed to the ability of E. coli to effectively cope with NFZ- and/or MMS-induced DNA damage (summarized in Table 1 & Fig 8A & 8B). The simplest explanation for these phenotypes is that these residues either contact the DNA, despite the fact that they failed to do so in the crystal, or that their substitution affects the structure of adjacent residues in the β clamp that do contact the DNA [27]. However, this argument fails to explain the MMS phenotypes of the loop I dnaN-L21A and dnaN-T26A mutants. Residue R24 is the only
position in loop I known to contact DNA, yet dnaN-R24A failed to exhibit MMS sensitivity. In contrast, dnaN-L21A and dnaN-T26A each conferred MMS sensitivity (summarized in Table 1 & Fig 8B), arguing it was independent of β clamp-DNA interactions. The dnaN-P20A, dnaN-L21A and dnaN-R24A mutants were slightly elevated for oriC relative to terC (summarized in Table 1 & Fig 8E). Although these differences relative to the wild type control were not statistically significant, they nevertheless suggest that loop I of the β clamp may contribute to hda function, possibly via a physical interaction.

In conclusion, 17 of 22 dnaN mutations examined in this work displayed one or more mutant phenotype. While it is possible that some of the mutations may affect interaction of the β clamp with one or more of its partner proteins, these findings nevertheless support a model in which β clamp-DNA interactions contribute to the ability of the clamp to manage the actions of its different partner proteins, particularly Pol IV, Pol V and Hda.

Supporting Information

S1 Fig. Molecular structure of the ΔdnaN::(kan, sacB) allele. Cartoon depiction of the wild type dnaA-dnaN-recF operon and ΔdnaN::(kan, sacB) allele. Nucleotide (kb) and minute (min) positions refer to approximate E. coli chromosomal coordinates. Approximate positions of homology for oligonucleotide primers used for construction of the ΔdnaN::(kan, sacB) allele and its recombineering onto the bacterial chromosome are indicated.

S2 Fig. Mutant dnaN strains fail to increase UV sensitivity. UV sensitivity of strains bearing mutations in (A) loop I (red), (B) loop II (blue) or (C) the central pore of the β clamp (green) was measured as described in Materials and Methods. This experiment was performed 4 times with 2 separate clones. Representative results shown.

S3 Fig. Mutant dnaN strains fail to increase H₂O₂ sensitivity. H₂O₂ sensitivity of strains bearing mutations in either loop I (red), loop II (blue) or the central pore of the β clamp (green) was measured as described in Materials and Methods. Results represent the average of 4 separate determinations ± one standard deviation. Sensitivity of the mutants was not significantly different than that of the dnaN⁺ wild type (WT) strain (p > 0.05).

S4 Fig. Ability of the dnaN-K12E mutant to support MMS-induced mutagenesis. The ability of the wild type (MN100-1 & MN100-2) and dnaN-K12E (MN114-1 & MN114-2) strains to support MMS-induced mutagenesis was measured as described in Materials and Methods. Results represent the average of 3 independent determinations using 2 separate clones ± one standard deviation. Symbols are as follows: *, p < 0.05; **, p < 0.001.

S1 Table. Bacterial strains and plasmids DNAs used in this study.

S2 Table. Oligonucleotides used in this study.

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Author Contributions
Conceptualization: VMPB MDS.
Data curation: MTN VMPB MAG MDS.
Formal analysis: MTN.
Funding acquisition: MDS.
Investigation: MTN VMPB MAG.
Methodology: MTN VMPB MAG MDS.
Project administration: MTN VMPB MAG MDS.
Resources: MTN VMPB MAG.
Supervision: MDS.
Validation: MTN VMPB.
Visualization: MTN MDS.
Writing – original draft: MDS.
Writing – review & editing: MTN VMPB MAG MDS.

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