STRUCTURE FUNCTION ANALYSIS OF *ESCHERICHIA COLI* DNA POL IV

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by

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ABSTRACT OF DISSERTATION

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Abstract

Translesion synthesis (TLS) DNA polymerases are low fidelity DNA polymerases responsible for inserting a deoxynucleotide opposite to and extending from replication-blocking DNA lesions that have evaded high fidelity DNA repair pathways. *E. coli* DinB (DNA Pol IV) is a TLS polymerase of great interest because of its conservation throughout all domains of life and its relatively high intracellular basal level when compared to other DNA polymerases. Alternative activities of DinB other than its TLS ability include: roles in recombination, transcription, and a DNA replication "checkpoint." Using a structure/function strategy, we sought to better understand these activities by utilizing *dinB* alleles with different catalytic activities, in combination with other mutated genes involved in DNA replication or repair. This approach has allowed us to identify residues of DinB important for its various cellular tasks. In this work we have identified the roles of three highly conserved catalytic residues in the fidelity of lesion bypass of diverse lesions.

We also addressed the underlying mechanisms governing interactions between different bacterial DNA polymerases upon DNA damage treatment, which is likely to activate either DNA polymerase exchange and/or DNA replication checkpoints. By using the *dnaE915* allele, encoding a variant of the replicative DNA Pol III catalytic α-subunit (Pol IIIα), we detected a DNA damage treatment-dependent growth arrest at *in vivo* DinB intracellular concentrations much lower than those in previous studies. A variety of intragenic *dinB* mutations localized to a specific area of DinB that suppressed any observed growth defects were also identified. We infer that this DinB face is interacting directly or indirectly with DNA Pol IIIα.
Lastly, we discovered a novel TLS independent role of DinB in the tolerance of DNA damage treatment. However, this role does not simply involve protein-protein interactions but requires the catalytic activity of DinB. Therefore, survival is likely the result of the largely error free synthesis of undamaged DNA that is independent of recA mediated recombination, induction of the SOS DNA damage response, or base excision repair. The structure/function analysis undertaken in this work has allowed us to discern and explore several activities of DinB other than its part in canonical DNA damage tolerance through strict TLS. This information will allow greater understanding of the mechanisms regulating both the TLS activity and other alternative roles that these specialized DNA polymerases might be playing in cells experiencing DNA damage or environmental stress.
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TABLE OF CONTENTS

Abstract 2
Acknowledgements 5
Table of Contents 6
Introduction 7
Chapter 1: An active site aromatic triad in *Escherichia coli* DNA Pol IV coordinates cell survival and mutagenesis in different DNA damaging agents. 12
Chapter 2: DNA damage and resulting functional interactions between *Escherichia coli* DNA Pol III and DNA Pol IV. 44
Chapter 3: DNA Pol IV plays a novel TLS independent role in cell survival upon DNA damage treatment in *Escherichia coli*. 86
Conclusions 104
Appendix A Chapter 1 strain, plasmid, and oligonucleotide list 111
Appendix B Chapter 2 strain, plasmid, and oligonucleotide list 113
Appendix C Chapter 3 strain, plasmid, and oligonucleotide list 117
Introduction

All cells accumulate DNA damage that, if left unrepaired, will stall DNA synthesis due to the inability of high fidelity DNA polymerases to use lesion-containing DNA as template [1]. DNA replication stalling is a potentially lethal event. To prevent death, cells respond to replication fork stalling by upregulating the expression of specialized DNA polymerases, which can perform translesion synthesis (TLS) consisting of both insertion opposite to and elongation from lesion-containing templates [2]. TLS is potentially a low fidelity process resulting in elevated mutagenesis. In *Escherichia coli* there are three TLS polymerases that are regulated by the SOS gene network, one of the cellular responses to replication stress [1, 3]. DinB (DNA Pol IV) is of particular interest because of its evolutionary conservation [1, 4, 5] and its high basal intracellular concentration (~250 nM) [1, 2, 6]. Indeed, this is approximately 17 fold higher [6] than that of DNA Pol III complex (the replicative DNA polymerase, 15 nM; [2]).

Strains lacking the *dinB* gene (ΔdinB) are sensitive to reagents that generate persistent DNA lesions on the N² group of deoxyguanine (N²-dG) such as nitrofurazone (NFZ) [7] as well as to alkylating agents such as methyl methanesulfonate (MMS) [8]. The introduction of a low copy number plasmid expressing *dinB* under the control of its native promoter is able to complement the NFZ or MMS sensitivity of ΔdinB strains [7-9]. DinB also has roles in recombination, transcription, and a DNA replication "checkpoint" [10-13].

We undertook a structure/function analysis approach in an attempt to address several questions concerning the importance of DinB in DNA replication. In Chapter 1 the roles of various amino acids in the DinB active site are examined. This work was
performed to determine whether the same active site residues are involved in the bypass or its fidelity of both alkylation and $N^2$-dG lesions. We hypothesized a highly conserved triad of aromatic residues (F12, F13, or Y79) [14, 15] were integral in TLS and we tested this by changing the aromatic triad residues to those of different polarity or size. We find these residues are all important for the in vivo bypass of either alkylating or $N^2$-dG lesions but that the relevance of specific residues in governing TLS fidelity is lesion dependent.

In Chapter 2 we addressed the underlying mechanisms governing interactions between different bacterial DNA polymerases upon DNA damage treatment, which is likely to activate either DNA polymerase exchange and/or DNA replication checkpoints. We set about analyzing a genetic system consisting of strains with both an antimutator dnaE allele (dnaE915) [16], encoding a variant of Pol IIIα, and dinB alleles with mutations in the aromatic triad. Because Pol IIIα(915) interacts differently with DinB compared to Pol IIIα, we hypothesized this system would permit us to detect in vivo interactions between DNA polymerases as lack of cell growth upon treatment with DNA damaging agents. We detected a DNA damage treatment dependent growth arrest at in vivo DinB intracellular concentrations much lower than that of previous studies [10]. A variety of intragenic dinB mutations localized to a specific area of DinB that suppressed any observed growth defects were also identified. We infer that this DinB face is interacting directly or indirectly with DNA Pol IIIα and to our knowledge, this is the first evidence identifying a region of the DinB protein functionally interacting with the replicative DNA Pol IIIα.
Chapter 3 addresses a novel TLS independent role for DinB in cellular survival upon DNA damage treatment. One explanation for the relatively high concentration of DinB in the cell when compared to other polymerases could be its role in the tolerance of endogenous DNA damage generated by normal metabolism [1]. We hypothesized that ∆dinB strains deficient in SOS induction (∆recA or lexA3) or base excision repair (BER; ∆alkA∆tag) would allow us to detect the changes in survival due to the replication of an undamaged template by DinB upon DNA damage treatment. To separate the importance of DinB’s replication of an undamaged template from its replication of a lesion containing template in damage tolerance, we utilized DinB(Y79A) or DinB(F13V) variants that are unable to bypass alkylating or N2-dG lesions, but retain catalytic activity on undamaged DNA. We find expression of dinB(Y79A) and dinB(F13V) but not dinB(D103N), an allele encoding a catalytically dead DinB, rescues these highly sensitive cells upon treatment with DNA damaging agents. This indicates catalytic activity, but not TLS activity, is required for cellular resistance to treatment. Furthermore ∆recA∆dinB cells with pdinB(Y79A), an allele shown to be a mutator upon NFZ treatment in recA+∆dinB cells, have a low mutant frequency upon treatment with NFZ or MMS. These results indicate DinB plays a TLS independent role in the cellular response to DNA damage that is solely due to the largely error-free synthesis of undamaged DNA that is independent of recA mediated recombination, induction of the SOS DNA damage response, or BER.

The structure/function analysis undertaken in this work has allowed us to discern and explore several activities of DinB other than its part in canonical DNA damage tolerance through strict TLS. We have analyzed catalytic residues important for TLS and
replication fidelity in the presence of DNA damage treatment. The conservation of these catalytic residues suggest what we learn in DinB can also be useful in understanding the activity of its homologues, such as human Pol κ (Chapter 1, Fig 1; [9]). We have shown a DinB-mediated DNA damage growth arrest/replication checkpoint can occur at near physiological levels of the protein and identified a face of the DinB protein important for affecting this growth arrest. Additionally we have opened the door for investigation into a previously unidentified role for DinB in TLS independent DNA synthesis upon DNA damage treatment. This information will allow greater understanding of the mechanisms regulating both the TLS activity and other alternative roles that these specialized DNA polymerases might be playing in cells experiencing DNA damage or environmental stress.

References

Chapter 1

An active site aromatic triad in *Escherichia coli* DNA Pol IV coordinates cell survival and mutagenesis in different DNA damaging agents.

Abstract

DinB (DNA Pol IV) is a translesion (TLS) DNA polymerase, which inserts a nucleotide opposite an otherwise replication-stalling $N^2$-dG lesion *in vitro*, and confers resistance to nitrofurazone (NFZ), a compound that forms these lesions *in vivo*. DinB is also known to be part of the cellular response to alkylation DNA damage. Yet it is not known if DinB active site residues, in addition to aminoacids involved in DNA synthesis, are critical in alkylation lesion bypass. It is also unclear which active site aminoacids, if any, might modulate DinB’s bypass fidelity of distinct lesions. Here we report that along with the classical catalytic residues, an active site “aromatic triad”, namely residues F12, F13, and Y79, is critical for cell survival in the presence of the alkylating agent methyl methanesulfonate (MMS). Strains expressing *dinB* alleles with single point mutations in the aromatic triad survive poorly in MMS. Remarkably, these strains show fewer MMS-than NFZ-induced mutants, suggesting that the aromatic triad, in addition to its role in TLS, modulates DinB’s accuracy in bypassing distinct lesions. The high bypass fidelity of prevalent alkylation lesions is evident even when the DinB active site performs error-prone NFZ-induced lesion bypass. The analyses carried out with the active site aromatic triad suggest that the DinB active site residues are poised to proficiently bypass distinctive DNA lesions, yet they are also malleable so that the accuracy of the bypass is lesion-dependent.
**Introduction**

Replicative DNA polymerases are multi-protein complexes responsible for synthesizing a high fidelity copy of a cell’s genome. Persistent lesions on the template DNA, which DNA repair pathways have failed to recognize, result in stalling of DNA replication, a potentially lethal event [1]. To avoid lethality, specialized DNA polymerases insert deoxynucleotides (dNTPs) opposite replication-blocking DNA lesions in a process known as translesion synthesis (TLS). This is largely a low fidelity process usually resulting in elevated mutagenesis [1,2]. In *Escherichia coli* there are three TLS polymerases that are regulated by the SOS gene network, one of the cellular responses to DNA damage and environmental stress [1,3]. The *polB* gene encodes the B family DNA Pol II, while the *dinB* gene and the *umuDC* operon encode the two Y family DNA polymerases, DNA Pol IV and DNA Pol V respectively [1,4,5,6,7]. DinB is of particular interest because of its evolutionary conservation [1,6,8] and its high basal intracellular concentration (~250 nM) [1,9,10]. Indeed, this is approximately 17 fold higher [10] than that of DNA Pol III complex (the replicative DNA polymerase, 15 nM; [9]) and is similar to that of the processivity clamp (α-clamp, 250 nM; [11,12]), an essential replication factor known to both recruit all DNA polymerases to the replication fork and manage their activity in the cell [13,14].

*E.coli* cells lacking the *dinB* gene (Δ*dinB*) are sensitive to nitrofurazone (NFZ) and 4-nitroquinoline-1-oxide (4-NQO) [15,16], reagents that create persistent DNA lesions on the N\(^2\) group of deoxyguanosine (N\(^2\)-dG) [17,18]. Recent evidence suggests that DinB and its homologues can also perform TLS of lesions that are the product of alkylation of DNA bases [19,20,21]. Alkylating agents are both a byproduct of the cell’s
metabolism and also come from diverse exogenous sources generating DNA damage in prokaryotic and eukaryotic cells [1,22,23,24,25]. In addition, alkylation agents are used as anti-cancer chemotherapeutic agents, [1,26,27], underscoring the significance of understanding the cellular mechanisms of alkylation lesion tolerance.

It is known that base excision repair pathways are the primary cellular response to alkylation damage [1,28,29,30,31,32], though Y family DNA polymerases are also part of this response [1,19,20,21]. These polymerases likely bypass 3-methyladenine (3-meA; [1,19,20,21,33,34]), a prevalent alkylation lesion that persists on the DNA and brings about replication fork stalling and cell death [20,21,35,36]. Indeed, *E. coli* strains lacking the *dinB* gene (Δ*dinB*) are sensitive to several alkylating agents, such as methyl methanesulfonate (MMS; [19]). Similar sensitivity is found in eukaryotic cells deficient in TLS polymerases [20,21]. Thus, the evidence so far indicates that if DNA repair pathways do not effectively recognize 3-meA, Y family DNA polymerases are critical in the cell's response to alkylation damage [19,20,21]. Unfortunately, 3-meA has a very short *in vitro* half-life [21,37], making difficult to directly investigate the bypass mechanisms of this alkylation lesion.

Most of our knowledge in regard to the active site of DinB has been acquired through studies with reagents that generate *N*²-dG lesions [15,16]. However, it is not known which aminoacids in the DinB active site are important for the bypass of alkylation lesions, e.g. most likely 3-meA. It is also unclear whether the same active site residues are involved in the bypass or its fidelity of both alkylation and *N*²-dG lesions. Structural modeling predicts that Pol κ (the mammalian DinB homologue) could
accommodate either the $N^2$-dG or 3-meA minor groove adducts in its active site in a conformation that would allow both insertion and extension from either adduct [20].

**Figure 1.** Homologous *E. coli* DinB and human Pol κ aromatic triads appear similarly positioned in the active site. The near identical conformation of the aromatic triads of (A) DinB (Pol IV) (F12, F13 and Y79) and (B) Pol κ (F111, Y112, and Y174) suggests these residues could be required for TLS activity of Pol κ. The DinB structure is from an *in silico* model generated in collaboration with A. Abyzov and V. Ilyin [40]. Image generated using PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA.). Pol κ structure was rendered using the pdb 3IN5 with PyMOL.

We studied a triad of aromatic residues (Fig 1) that is conserved in Y family DNA polymerases including Pol η and Pol κ [38,39]. Here, we describe the analysis of the aromatic triad residues of DinB in response to DNA damage generated by treatment with MMS or NFZ, reagents that create respectively alkylation or $N^2$-dG lesions *in vivo*. This report describes the effect of changing the aromatic triad residues to those of different polarity or size on both survival and DNA damage-induced mutagenesis. We used the strictly catalytic aspartic acid 103 (D103) that is known to be critical for DNA synthesis and thus unable to complement a ΔdinB strain as a control [16,40,41]. This type of analysis has permitted us to learn about the intricacies of *in vivo* DinB lesion bypass
activities. We infer that the classical catalytic and the highly conserved DinB active site “aromatic triad” are necessary for TLS of alkylation lesions. Remarkably, the aromatic triad also serves the function of governing in vivo TLS fidelity, which seems to be lesion-dependent.

Results

DinB active site residues are important for survival in MMS.

The two catalytic activities of DinB, phosphodiester bond formation (i.e. DNA synthesis) and lesion bypass are separable [16]. Each activity can be tested in vivo by measuring survival of cells lacking the chromosomal copy of the dinB gene (∆dinB) after treatment with a DNA damaging agent. Because some alkylation lesions are chemically unstable (e.g. 3-meA), we took advantage of this genetic approach to determine whether ∆dinB cells expressing plasmid-borne dinB alleles with mutations in the aromatic triad could survive MMS treatment.

Figure 2. Kinetic of MMS lethality of ∆dinB strains harboring either dinB⁺ or catalytic/translesion deficient dinB alleles. Only the plasmid-borne dinB⁺ allele rescues ∆dinB MMS sensitivity. Neither plasmid-borne DinB(D103N) nor DinB(F13V) rescue ∆dinB cells treated with various concentrations of MMS. Enhanced sensitivity is observed in ∆dinB strains expressing DinB(D103N) when compared to ∆dinB. Error bars represent the standard deviation of the mean from at least 3 independent experiments.
Low copy number plasmids expressing different \textit{dinB} alleles from the native SOS inducible promoter were introduced into \textit{ΔdinB} by transformation (Appendix A). Cells were then assayed for survival at various concentrations of MMS. As expected, the plasmid carrying native \textit{dinB}+ rescues \textit{ΔdinB} treated with MMS (Figs 2 and 3), while the strain expressing the catalytically inactive \textit{dinB(D103N)} variant [41] is highly sensitive to MMS treatment (Figs 2 and 3). Unexpectedly, \textit{ΔdinB} cells expressing \textit{dinB(F13V)} did not show the prototypical highly NFZ sensitive phenotype [16] upon treatment with MMS (Figs 2 and 3).

We were intrigued by the enhanced cellular sensitivity to MMS (\textit{ΔdinB} with the \textit{dinB(D103N)}, gray bars; Fig 3) or NFZ (\textit{ΔdinB} with either the \textit{dinB(D103N)} or \textit{dinB(F13V)}, white bars; Fig 3) compared to \textit{ΔdinB}. The simplest explanation is that the phenotype is due to high DinB intracellular concentrations, despite being expressed under the SOS-regulated native promoter and from low copy number plasmids. Increased

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{\textit{ΔdinB} is rescued from MMS and NFZ lethality only by \textit{dinB}+. (A) Neither plasmid-borne DinB(D103N) nor DinB(F13V) rescue \textit{ΔdinB} cells treated with MMS (7.5 mM) or NFZ (0.008 mM). Enhanced sensitivity to MMS is observed in \textit{ΔdinB} with DinB(D103N) (gray bars). \textit{ΔdinB} strains with either DinB(F13V) or DinB(D103N) variants exhibit an enhanced cellular sensitivity to NFZ compared to \textit{ΔdinB} (pVector, white bars) as previously reported [16]. Error bars represent the standard deviation of the mean from at least 3 independent experiments.}
\end{figure}
intracellular concentrations of DinB variants may somehow have a more deleterious effect on survival than lack of DinB. Thus, the \( \text{dinB(D103N)} \) and \( \text{dinB(F13V)} \) alleles (Appendix A) were crossed onto the chromosome replacing \( \text{dinB}^+ \), as indicated in Materials and Methods.

Consistent with the hypothesis, we find that cells with a chromosomal copy of \( \text{dinB(D103N)} \) are no longer highly sensitive to MMS (compare ~100 fold more killing than \( \Delta \text{dinB} \) in Fig 3 with the same lethality as \( \Delta \text{dinB} \) in Fig 4) or NFZ at any of the concentrations tested, and survive treatment as \( \Delta \text{dinB} \) cells (Fig 4). Conversely, cells with \( \text{dinB(F13V)} \) in the chromosome remain more sensitive to NFZ than \( \Delta \text{dinB} \), though the extent of sensitivity is less dramatic in the chromosome (~10 fold, white bars; Fig 4). These data demonstrate that the observed exacerbated sensitivity phenotypes of \( \Delta \text{dinB} \) strains with plasmid-borne

**Figure 4.** MMS or NFZ survival phenotypes of strains with catalytic or TLS deficient dinB chromosomal alleles. Cells carrying a single chromosomal copy of the catalytic deficient \( \text{dinB(D103N)} \), the TLS deficient \( \text{dinB(F13V)} \), and other \( \text{dinB} \) alleles were assayed for survival as indicated in material and methods with MMS (7.5 mM) or NFZ (0.008 mM). Error bars represent the standard deviation of the mean from at least 3 independent experiments.
TLS deficient *dinB* alleles are not solely due to elevated intracellular concentrations of these proteins. However, the mechanism(s) underlying this phenomenon is/are not fully understood.

**The aromatic triad is required for *in vivo* DinB TLS.**

In an effort to gain insights into the TLS activity of DinB in alkylation lesion bypass, we looked for conserved residues in the DinB active site that could be as important as F13 in DinB *N*²-dG TLS. This analysis, carried out with a large number of

![Figure 5](image)

**Figure 5. An aromatic triad in the DinB active site is required for ΔdinB survival upon treatment with MMS or NFZ.** ΔdinB harboring the dinB alleles with mutations at position 12, 13, and 79 were treated with MMS (7.5 mM; gray bars) or NFZ (0.008 mM; white bars). Treatments were carried out as described in materials and methods. Error bars represent the standard deviation of the mean from at least 3 independent experiments. Only top error bars are shown for clarity.
DinB sequences (>100) in both prokaryotic and eukaryotic organisms (including the DinB human homologue Pol κ), surprisingly shows that phenylalanine 13 (F13) is only somewhat conserved (42%). However, if the analysis also considers tyrosine, a structurally similar residue, then F13/Y13 becomes 97% conserved. We hypothesized that the aromatic ring of tyrosine or phenylalanine fulfills an identical role in lesion bypass. We constructed a DinB derivative with a tyrosine at position 13 instead of phenylalanine (pdinB(F13Y)) in the same low copy number plasmid mentioned in the above section, and introduced it by transformation into ΔdinB. As predicted, the ΔdinB/pdinB(F13Y) strain has the same level survival after MMS or NFZ treatment as cells with wild-type DinB⁺ (Fig 5).

We have shown (Figs 2, 3 and 4) that F13 is important for the TLS of alkylation lesions, but strains expressing this allele survive better in MMS than in NFZ regardless of the allele location (Figs 3 and 4). F13 was also changed to alanine or serine. Unlike dinB(F13V), both dinB(F13A) (data not shown) and dinB(F13S) (Fig 5) result in decreased survival of ΔdinB cells upon NFZ or MMS treatment independent of whether the dinB alleles are expressed from a plasmid (Fig 5) or from the chromosome (Fig 4). It can be inferred from these data that the role of the F13 residue in the DinB bypass of alkylation DNA lesions is likely different from its role in N²-dG bypass, but is nevertheless essential for the bypass of MMS-derived lesions in vivo.

The two other aromatic residues that are also highly conserved (>95% conservation among DinB sequences) and happen to be in close proximity to F13 in the DinB tertiary structure are Y79 and F12 (Fig 1). The conservation is true even at the structural level (Fig 1, compare DinB and Pol κ) suggesting that, unlike F13, both the
aromatic ring and the polarity of these residues might be equally important for lesion bypass.

We changed each one of these residues, and assessed their function by measuring survival of \( \Delta \text{dinB} \) carrying the various \( \text{dinB} \) alleles upon MMS or NFZ treatment. Y79 or F12 were changed to the aromatic residue with the opposite polarity (phenylalanine or tyrosine respectively) or to the non-aromatic residues alanine or serine.

There is no measureable survival defect for the \( \Delta \text{dinB} \) strain upon MMS treatment when the conserved Y79 residue is replaced by phenylalanine (Fig 5). However, \( \Delta \text{dinB} \) cells expressing \( \text{dinB}(Y79A) \) show an enhanced sensitivity to MMS or NFZ compared to \( \Delta \text{dinB} \) (pVector; Fig 5). A similar enhanced sensitivity, though not to the same degree, was observed in NFZ-treated cells when the \( \text{dinB}(Y79A) \) allele is expressed from the chromosome (Fig 4). In contrast, the result of exchanging the tyrosine for a non-aromatic amino acid of the same polarity, serine, leads to poor survival in MMS or NFZ treatment, similar to that shown by the \( \Delta \text{dinB} \) strain (Fig 5).

\( \Delta \text{dinB} \) expressing the plasmid-borne \( \text{dinB}(F12Y) \) allele, however, show reduced survival in MMS or NFZ when compared to cells carrying \( \text{p} \text{dinB}^+ \), but survive better than \( \Delta \text{dinB} \), suggesting that TLS is lessened but not abolished in this variant (Fig 5). This is confirmed by changing F12 to alanine (Fig 5), which results in survival similar to that of cells lacking \( \text{dinB} \). We also investigated the effect of changing F12 to a non-aromatic residue of the opposite polarity and found that the F12S mutation does abolish TLS activity in vivo, similar to the F12A mutation (data not shown).

Taken together, the data demonstrate that the aromatic triad consisting of F12, F13, and Y79 are all needed for survival in MMS. The relevance of each residue in TLS
varies depending on the lesion and is independent of the location (plasmid or chromosome) of the dinB TLS deficient allele. Importantly, the absolute requirement of

these residues for DinB lesion bypass, and the evolutionary conservation of these aromatic residues, suggest the importance of corresponding residues in DinB homologues, such as human Pol κ (Fig 1).
Survival effects of various dinB alleles expressed in ΔdinB depend on both the lesion and the DinB processivity clamp-binding motif.

We asked whether the survival phenotypes of ΔdinB expressing various DinB variants were independent of the DNA damaging agent used to treat cells. We took advantage of the enhanced sensitivity phenotype observed in ΔdinB strains such as those carrying plasmid-borne dinB(D103N), dinB(F13S), and dinB(Y79A) (to MMS or NFZ), or dinB(F13V) (to NFZ). We have already shown (Fig 4) that the increased sensitivity of the ΔdinB/pdinB(D103N) strain to MMS or NFZ compared to ΔdinB, is likely due to elevated intracellular concentrations. Nonetheless, when compared to ΔdinB, expression of the dinB(F13S), dinB(Y79A), or dinB(F13V) alleles results in enhanced sensitivity to DinB cognate lesions regardless of whether they are expressed from a plasmid or from the chromosome (see Figs 3, 4, and 5). Therefore, if survival upon MMS or NFZ treatment is TLS independent, dinB(F13V), and especially dinB(D103N) should render ΔdinB strains sensitive to any DNA damaging agent regardless of the lesion it might bring about. UV damage was chosen to test this model because DinB is unable to bypass the thymine-thymine dimer lesions generated in the major groove of the DNA upon treatment [42]. 37 J/m² of UV light, a dosage at which ΔdinB or dinB⁺ strains are equally killed was used to treat ΔdinB strains with these dinB alleles. We find that ΔdinB cells bearing either the dinB catalytic or TLS deficient alleles survive as well as ΔdinB or dinB⁺ strains upon UV treatment. In contrast, at comparable levels of MMS or NFZ treatment, ΔdinB strains expressing DinB(D103N) or DinB(F13V), show enhanced sensitivity to MMS or NFZ (Fig 6). Thus, only DinB cognate lesions result in poor
survival of ΔdinB cells expressing these dinB alleles, suggesting that survival in MMS or NFZ treatment is linked to TLS.

We next investigated whether the DinB variant-mediated enhanced sensitivity in MMS or NFZ requires the carboxy terminal residues known to interact with the processivity factor β-clamp [43]. Thus, derivatives of DinB(F13V), DinB(D103N), and DinB⁺, as a control, lacking the DinB β-clamp binding motif (347LVLGL351; [43,44]) were constructed in the same low copy number plasmids (Appendix A). If ΔdinB strains expressing DinB variants lacking the β-clamp binding motif are as sensitive to either MMS or NFZ as ΔdinB, then it could be inferred that the observed enhanced sensitivity is mediated through interactions with the β-clamp, and is consistent with the idea that these DinB variants are localized at the replication fork. We found that ΔdinB with pdinB⁺Δβ are more sensitive to MMS or NFZ compared to pdinB⁺ (Fig 7). In contrast, ΔdinB cells

Figure 7. ΔdinB with variants lacking the residues comprising the β-clamp binding motif are no longer highly sensitive to MMS or NFZ. (A) A representative LB medium plate containing MMS (7.5 mM) with 10 fold serial dilutions of dinB cells bearing the plasmid-borne dinB alleles is shown. (B) Same as (A) except cells were deposited on LB medium plates with NFZ (0.008 mM).
expressing DinB(D103N)Δβ on MMS or NFZ (Fig 7) and those expressing DinB(F13V)Δβ on NFZ (Fig 7B) are more resistant to these DNA damaging agents. These data suggest that the enhanced sensitivity observed with these dinB alleles is dependent on the β-clamp binding motif. This is perhaps the result of the interaction of DinB with the β-clamp, which is likely to be occurring at the replication fork.

From these independent sets of data we can deduce that the in vivo phenotypes observed in cells expressing dinB catalytic or other dinB alleles are the result of in vivo deficiencies in specific lesion bypass.

**DNA damage-induced mutation frequency as a measure of accurate TLS activity.**

In this report we present a DNA damage-induced mutagenesis screen with a substantial mutational target size [45,46]. Cells are treated with either MMS or NFZ at concentrations where ΔdinB cells are equally killed, i.e. 7.5 mM MMS and 0.008 mM NFZ. Bacterial colonies that survive the treatment are then screened for loss of growth in minimal medium. A very conservative estimation of the target size is between 35 and 100 Kb, since any mutation that results in the inability to grow in minimal medium will be scored as a mutant. These include genes involved in amino acid, vitamin, or nucleotide biosynthesis. There is no selection in the detection of the mutant population that arises as the result of DNA damage and mutant colonies unable to grow in minimal medium are clonal.

We find that there is virtually no DNA damage-induced mutagenesis in ΔdinB expressing dinB⁺ (Fig 8). Importantly, although DinB is arguably at higher intracellular concentration than when expressed from a single chromosomal copy, it does not increase DNA damage-induced mutant frequency in this assay simply by being at a higher
Figure 8. DNA damage-induced mutants. (A) ΔdinB with plasmid-borne DinB variants were treated with MMS (7.5 mM), NFZ (0.008 mM), or UV light (37 J/m²) and screened for mutants unable to grow on glucose minimal medium. Only ΔdinB strains carrying plasmid-borne dinB⁺, dinB(D103N), dinB(F13V), dinB(F13S), or dinB(Y79A) alleles were assessed for UV-induced mutants. NFZ-induced mutants were also ascertained in the dinB(Y79A) strain in which the DinB variant is expressed from the chromosome (bold font). Mutants were equally distributed when independent cultures were analyzed. All samples have a standard error ≤ 5% of the average of mutants obtained per individual culture. (B) The fold difference shown in mutants is relative to ΔdinB.
intracellular concentration. We also found low frequencies of mutants in ΔdinB and in ΔdinB expressing the catalytically inactive dinB(D103N) (Fig 8), both of which are presumed to be the consequence of an activity independent from DinB.

Cells expressing dinB with mutations in the aromatic triad residues F13, Y79, and F12 display a frequency of MMS-induced mutants similar to ΔdinB or to the ΔdinB/pdinB(D103N) strain (Fig 8). We find that ΔdinB cells expressing the DinB(F13V) variant have a modest increase in both MMS and NFZ-induced mutant frequency when compared to either ΔdinB or to those expressing DinB(D103N) (Fig 8). Unexpectedly, there is a substantial increase in mutants for NFZ-treated ΔdinB strains expressing pdinB(F13S), pdinB(Y79A), or pdinB(Y79S) (Fig 8). Notably, this increase is also observed in the dinB(Y79A) chromosomal strain (Fig 8; bold font).

We also assessed the number of UV-induced mutants to validate the level of mutagenesis that is DinB-independent. We expected that UV-induced mutant frequencies would be similar to ΔdinB or ΔdinB strains expressing the catalytically inactive derivative DinB(D103N) after NFZ or MMS treatment. This is what was observed (Fig 8) in the cases where it was determined. Intriguingly, we observed an increase in UV-induced mutants in strains expressing pDinB+ but not pDinB(D103N) (compare columns NFZ or MMS with UV for pDinB+ in Fig 8A). This has been previously reported for DinB+ in an independent experiment in which selection for UV-induced Arg+ mutants was carried out [40]. Thus, there might be a role for DinB, or more likely DinB with its interacting partners [40], in regulating this mutagenesis.

**MMS induces the SOS-gene network more strongly than NFZ.**
The difference between NFZ- and MMS-induced mutant frequencies might be due to a fundamental distinction between the mechanisms regulating DinB alkylation or $N^2$-dG lesion bypass. Perhaps there are other SOS induced proteins that might explain the elevated mutant frequency observed exclusively upon NFZ treatment. To measure the relative induction of the SOS gene network in cells treated with MMS or NFZ, $dinB^+$, $ΔdinB$, and $ΔrecA$ strains carrying a plasmid that expresses GFP from an SOS inducible promoter ($sulA$-GFP) were treated with MMS (7.5 mM), NFZ (0.06 mM shown), or the strong SOS inducer ciprofloxacin (Cip) (0.1 µg/mL) [49]. $dinB^+$ and $ΔdinB$ strains display a significant 50% increase, compared to the control $ΔrecA$ strain, in the ratio of Fluorescence/OD$_{600}$ after 2 hours of NFZ treatment and after 1.5 hours of both MMS and ciprofloxacin treatments, 5 hours is shown. For 20 hour kinetic see Fig S2. No increased fluorescence is detected in NFZ concentrations below 0.06 mM (data not shown) when compared to untreated $dinB^+$ and $ΔdinB$ cells, or the $ΔrecA$ negative control during the time frame the experiment was carried out (see Fig 10 for 20 hour kinetic). Error bars represent the standard deviation of the mean from at least 4 replicates.

Figure 9. Relative induction of the SOS gene network in cells treated with MMS or NFZ. $dinB^+$, $ΔdinB$, and $ΔrecA$ strains carrying a plasmid that expresses GFP from an SOS inducible promoter ($sulA$-GFP) were treated with MMS (7.5 mM), NFZ (0.06 mM shown), or the strong SOS inducer ciprofloxacin (Cip) (0.1 µg/mL) [49]. $dinB^+$ and $ΔdinB$ strains display a significant 50% increase, compared to the control $ΔrecA$ strain, in the ratio of Fluorescence/OD$_{600}$ after 2 hours of NFZ treatment and after 1.5 hours of both MMS and ciprofloxacin treatments, 5 hours is shown. For 20 hour kinetic see Fig S2. No increased fluorescence is detected in NFZ concentrations below 0.06 mM (data not shown) when compared to untreated $dinB^+$ and $ΔdinB$ cells, or the $ΔrecA$ negative control during the time frame the experiment was carried out (see Fig 10 for 20 hour kinetic). Error bars represent the standard deviation of the mean from at least 4 replicates.
fluorescence is thus an indicator of SOS induction. This plasmid was introduced into both $\text{dinB}^+$ and $\Delta\text{dinB}$ strains by transformation. In this experiment ciprofloxacin [49] instead of UV irradiation was chosen as the SOS inducer to directly evaluate GFP fluorescence in a comparable time frame.

We find that expression of GFP upon treatment with a DNA damaging agent is $\text{dinB}$ independent (Figs 9 and 10). Strikingly, we find that GFP fluorescence is greater in cells treated with MMS than in those treated with NFZ (Figs 9 and 10).

**Figure 10. MMS is a more robust inducer of the SOS response than NFZ.** Kinetic of the ratio of fluorescence over OD$_{600}$ is shown for $\text{dinB}^+$, $\Delta\text{dinB}$, and $\Delta\text{recA}$ strains carrying psul4p-GFP. Strains were treated with MMS (7.5 mM), NFZ (0.06 mM shown), or Cip (0.1 µg/mL). Fluorescence readings and optical density (600nM) were taken every 5 minutes for 20 hours in a plate reader. Data shown are the average of at least 4 replicates, and the standard deviation of the mean is ≤ 25% for all samples.
Moreover, no fluorescence was detected at NFZ concentrations lower than 0.06 mM, the concentration depicted in Fig 9. Yet the NFZ concentration used to treat cells throughout this report is 7.5 times lower i.e. 0.008 mM, an NFZ concentration insufficient to induce the SOS response at levels similar to those measured in MMS treatment. Thus, it can be inferred that the large number of NFZ-induced mutants is specific to the \(N^2\)-dG lesions and not due to overexpression of any other SOS-induced activity.

Taken together, this evidence indicates that the aromatic triad residues play different and nuanced roles in the TLS of MMS- and NFZ-induced lesions. The analyses carried out suggest that the active site is pliable and that the aromatic triad is essential for both bypass and accuracy.

**Discussion**

Much has been learned about the Y family translesion (TLS) DNA polymerase DNA Pol IV (DinB) in *E. coli* [1,6,50]. This DNA polymerase inserts a nucleotide opposite specific DNA lesions (i.e. bypass or translesion synthesis activity) with relatively high accuracy compared to other Y DNA polymerases [15,16]. Though under some conditions DinB has been shown to cause -l frameshift mutations on misaligned templates [40,51,52], this appears to be regulated by protein-protein interactions [40,51]. However, knowledge is lacking in regard to the role played by DinB’s active site residues, in alkylation lesion bypass. Moreover, it is not known whether residues in the active site play a role in the accuracy of bypass of distinct DNA lesions. To fill this knowledge gap, we have undertaken structure/function analyses of the DinB active site and have gained insights into the active site residues that govern bypass and fidelity of
different lesions. In the experiments reported here we use MMS and NFZ, reagents known to cause DNA lesions that kill cells without DinB [16,19]. Specifically, we studied an aromatic residue triad F12, F13, and Y79 in the DinB active site (Fig 1) by changing these conserved residues to ones of different size and polarity. Notably, this is the first report in which a number of these DinB variants have been studied when expressed from the chromosome.

The F13 residue is critical for both \textit{in vivo} and \textit{in vitro} DinB mediated bypass of $N^2$-dG lesions [16]. Agreeing with previously published reports [16], expression of the DinB(F13V) variant in Δ\textit{dinB} cells from a low copy number plasmid causes enhanced NFZ sensitivity (Fig 3). This phenotypic signature is remarkably maintained when this \textit{dinB} allele is expressed from the chromosome (Fig 4). Notably, there is no enhanced sensitivity to MMS (Figs 3 and 4), suggesting that the DinB active site adjusts to lesions. Thus, the relevance of different catalytic residues for bypass activity is likely to be lesion dependent.

We investigated whether changing the aromatic triad residues to different aromatic residues compromised the bypass activity of \textit{E. coli} DinB. Our results show that the polarity of the aromatic residue is important for the F12 residue but not for the others (Fig 5). It can be inferred that either a phenylalanine or a tyrosine at the F13 or Y79 positions allows for the insertion of a nucleotide opposite an $N^2$-dG or an alkylation lesion, resulting in no change in the activity of the DNA polymerase. A comparable result has been found in B-family DNA polymerases [53,54] when a similarly positioned residue was changed from a tyrosine to a phenylalanine. Based on the analysis carried out in Dpo4 [55], it is probable that the DinB(F12Y) mutation reduces hydrophobic packing,
which in turn leads to reduced TLS; however, in the Y79F mutation, the phenylalanine is able to stabilize the F13 residue allowing for efficient TLS. A recent study demonstrated that the active site residue Y112 (F13 in DinB) of the human DinB counterpart, Pol κ, is required not only for effective bypass of certain lesions and exclusion of rNTPs from DNA synthesis, but also for mismatch-primer extension [56]. It is plausible that mutations in either F111 (F12) or Y174 (Y79) could abolish the ability of Pol κ to carry out any of these functions.

We further analyzed these three key aromatic residues by assaying survival of ΔdinB strains bearing plasmid-borne DinB variants in which each of the aromatic triad residues were mutated to amino acids without aromatic rings. None of these variants rescued ΔdinB strains upon MMS or NFZ treatment, a phenotype that was maintained independently of the allele location (Figs 4 and 5), suggesting that all three aromatic residues are essential for bypass of both alkylation lesions and N²-dG minor groove adducts.

It was plausible that poor survival in NFZ or MMS by ΔdinB cells expressing the various dinB deficient alleles might be due to increased intracellular DinB concentrations [57] and not necessarily to TLS. If the variants were causing lethality due to, for example, unregulated access to stalled replication forks; it should be observed independent of both the treatment and the dinB deficient allele tested. However, this is not the case (Fig 6). Provocatively, the poor survival phenotype is only observed when cells are treated with either NFZ or MMS, but not with UV (Fig 6). We (Fig 3), and others [16,19] have shown that DinB is necessary for survival in alkylation or N²-dG DNA damage, demonstrating that MMS- and NFZ-induced lesions are cognate DinB lesions, i.e. DinB activity is
critical for survival. The same is true for other DinB-like polymerases [20,21].

Differences in survival are only observed when specific cognate lesions are present on the DNA, suggesting that lesions might actively recruit DinB polymerases to the replication fork, possibly increasing the local polymerase concentration. The localized concentration of DinB would then permit efficient exchange with the replicative polymerase, probably via the β-clamp [58]. This suggests that lesion specificity might play an important role in the TLS activity of DinB. This concept of lesion-induced recruitment of Y family DNA polymerases is similar to that occurring during somatic hypermutation ([59] and references therein).

Importantly, the SOS gene network is robustly induced with MMS but not so with NFZ. Indeed, at least a 7.5 fold higher NFZ concentration than the one used here to routinely treat cells with was required to detect any SOS induction (Fig 9). Interestingly, nitrofurantoin, another reagent within the class of activated nitrofurans [60] is also a poor inducer of the SOS response [61,62,63]. Thus, intracellular concentrations of DinB and other SOS-induced proteins would be higher in MMS than in NFZ treated cells. These results suggest that it is DinB and not other SOS induced proteins that are responsible for the observed loss in survival of ΔdinB cells expressing dinB catalytic or TLS deficient alleles (Figs 3, 4, and 5). Moreover, the phenotypic signature of some of these deficient alleles is maintained when crossed onto the chromosome (Fig 4) suggesting that it is not exclusively due to high intracellular concentrations. Finally, the DinB protein appears to be properly localized at or near the replication fork since cells carrying the dinB(F13V)Δβ or dinB(D103N)Δβ alleles, which lack the conserved motif that permits DinB-β clamp interactions, are no longer highly sensitive to MMS or NFZ (Fig 7). From these data we
can infer that DinB and its variants have to be at or near the replication fork to effect either survival or lethality of cells upon treatment with reagents producing DinB cognate lesions.

The analysis we have carried out indicates the aromatic triad in the active site of DinB is needed for cells to survive NFZ or MMS treatment because mutations in the aromatic triad impair \textit{in vivo} TLS.

Additionally, we report here that the aromatic triad is important for the accuracy of DinB bypass. The MMS- or UV-induced mutant frequency is quite low for \textit{ΔdinB} strains carrying any of the plasmid-borne DinB variants. Conversely, an NFZ-induced mutator phenotype is apparent in \textit{ΔdinB} cells carrying the DinB(Y79A), DinB(Y79S), or DinB(F13S) derivatives. A similar result is obtained with strains in which DinB(Y79A) is expressed from the chromosome (Fig 8). There are two possibilities as to why only NFZ induced mutagenesis is observed: (1) low fidelity on undamaged DNA, as some of these variants are known to be incapable of TLS \textit{in vitro} \cite{15,16}, and/or (2) mutagenesis is the product of the \textit{in vivo} TLS activity of these DinB variants. It is possible the \textit{in vitro} and \textit{in vivo} properties observed in DinB(Y79) variants are due to the inability of the new residue at position 79 to properly support F13 in carrying out high fidelity bypass. Although the substitution of valine for phenylalanine at position 13 does render DinB TLS deficient, it is only when a smaller serine is substituted for phenylalanine that the fidelity of the polymerase is severely compromised. All of these changes in either the F13 or Y79 do not affect the bypass fidelity in MMS, presumably because the active site is flexible and adapts to the different lesions. Perhaps the fidelity of alkylation lesion bypass is regulated differently from the fidelity of NFZ-induced lesion bypass.
The observed NFZ-induced mutants are also likely DinB dependent. If the mutants were occurring as a result of the action of another polymerase, i.e. DNA Pol V, it would have been evident in the UV-induced mutagenesis assay. UV treated cells expressing DinB variants, display a number of DNA damage-induced mutants that are, for the most part, equivalent to the level of mutants found both in ΔdinB carrying the DinB(D103N) variant, which is unable to synthesize DNA, and to cells without DinB (Fig 8). Furthermore, there is evidence that DNA Pol II is not involved in the bypass of MMS- or NFZ-derived lesions in cells that are proficient for base or nucleotide excision repair [19,64], making it difficult to envision a simple model in which DNA Pol II is responsible for the observed DNA damage-induced mutagenesis. We carried out Illumina deep sequencing of the genome of several independent MMS treated ΔdinB/pdinB(D103N) or pdinB(F13V) strains that we identified as mutants based on their inability to grow in minimal medium. Strikingly, MMS-induced mutants of ΔdinB/pdinB(D103N) have only single base pair substitutions (SNPs), including those in genes which could be responsible for the lack of growth in minimal medium (data not shown). Notably, both SNPs and -1 frameshifts (the mutational signature of DinB(F13V) [40]) were detected in mutants derived from ΔdinB/pdinB(F13V) strains. This evidence further suggests that DNA Pol IV and its variants are responsible for effecting mutagenesis.

There is growing evidence for a role of DinB-like polymerases in human cancers [65,66,67,68]. Thus, this triad of aromatic residues in the DinB active site might be playing similar roles in DinB homologues especially regarding fidelity. When compared to the in silico model of DinB, the Pol κ crystal structure shows that the aromatic triad is
identical in conformation (DinB F12, F13 and Y79 are homologous to Pol κ F111, Y112, and Y174; Fig 1). Notably, in the 1000 Genomes database [69] we find that there is only one known polymorphism in the protein sequence of Pol κ that is homologous to E. coli DinB, (S423R), which is not an active site residue. The lack of variations in the Pol κ sequences, especially in the active site, agrees with data showing natural populations of E. coli select against polymorphisms in the DinB catalytic domain [70]. The mutator phenotypes observed in cells expressing DinB(F13S, Y79S, or Y79A) also indicate that variations in homologous residues of Pol κ could lead to a similar reduction in TLS fidelity. The lack of polymorphisms in humans might also be due to selection against such changes, perhaps the result of embryonic lethality.

Thus, the analyses of the DinB active site and its aromatic triad have provided insights into mechanisms that govern both TLS and the fidelity of the bypass of different cognate lesions. In this regard, we found a strikingly low level of DNA damage-induced mutants in ΔdinB cells expressing wild type DinB from a low copy number plasmid, despite it both being at a higher copy number than chromosomal, and having a sizable mutational target. Furthermore, we found few alkylation DNA damage-induced mutants, in agreement with previous findings [19]. Finally, we found that the aromatic triad plays a key role in the bypass fidelity of NFZ-induced lesions. This supports the notion that N2-dG lesions might indeed be the preferred lesions recognized and bypassed by this DNA polymerase. Although bacteria may encounter NFZ as an antibiotic in the treatment of infections [71], we are still left with the question: what is the endogenous source of N2-dG lesions? We have no direct answer yet to this question, however, since DinB-like DNA polymerases are evolutionarily conserved, the source of their preferred N2-dG
lesion substrate must be the result of an ordinary metabolite. One exciting candidate is methylglyoxal, a byproduct of glycolysis that can form $N^2$-(1-carboxyethyl)-2’-deoxyguanosine ($N^2$-CEdG) lesions that are bypassed by DinB and human Pol $\kappa$ [72].

We have shown here that the high fidelity of DinB is apparent upon alkylation damage, an inescapable and pervasive form of DNA damage, even when the DinB active site performs in vivo error-prone NFZ-induced lesion bypass. In addition, we propose that it is the nature of the lesion that localizes DinB to the replication fork and facilitates protein-protein interactions to prompt DNA polymerase exchange with the replicative DNA polymerase when it has stalled.

**Materials and Methods**

**Bacterial Strains and Plasmids.** Bacterial strains and plasmids used in this report are listed in Appendix A. The P90C $\Delta$dinB strain was generated by P1 transduction using an allele from the KEIO collection [73] (a kind gift of the Lewis lab at NEU). Plasmid-borne DinB mutants were constructed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and introduced into CaCl$_2$ chemically competent cells by transformation [74]. Mutagenic oligonucleotides are listed in Appendix A. Mutations were verified by DNA sequencing, carried out at the Tufts University Core Facility in Boston, MA.

**Survival Assays.** Cultures were grown to saturation in either liquid LB or M9 minimal medium [74] with ampicillin (Amp, 200 $\mu$g/mL; Sigma). Serial dilutions of saturated cultures were treated with varying concentrations of methyl methanesulfonate (MMS, 5, 7.5, and 10 mM; Acros Organics), nitrofurazone (NFZ, 0.008 mM; Sigma), or were irradiated in minimal medium at a UV (254 nm) intensity of 37 J/m$^2$. 
Construction of DinB Variants in the Chromosome. *dinB(D103N), dinB(F13V), dinB(F13S),* and *dinB(Y79A)* alleles were introduced into the chromosome using the SOE-LRed method [75].

**Mutation Assays.** Cells were evenly spread with glass beads onto LB medium with 7.5 mM MMS or 0.008 mM NFZ. These concentrations of MMS and NFZ equally killed Δ*dinB* cells. UV irradiation (~37 J/m^2_) was carried out on M9 minimal medium supplemented with casaminoacids [45]. Under these conditions, this level of irradiation killed Δ*dinB* and *dinB*^+^ cells to the same extent. Surviving colonies were screened for loss of function on minimal medium without amino acid supplementation [45], except for proline [76], which is required by the parental strain. Three independent experiments were carried out per DinB derivative until a minimum of 1000 colonies were screened.

**SOS Induction Assays.** Cells bearing a plasmid expressing GFP from a *sulA* promoter (pUA66-*sulA*, [47]) were grown to saturation in minimal medium with Kanamycin (35 µg/mL; Sigma). These cultures were diluted 1 to 10 in the same growth medium with the appropriate concentration of MMS (7.5 mM), NFZ (0.008-0.06 mM), or ciprofloxacin (0.1 µg/mL, Cip; Sigma) in 96 well black plates with clear flat bottom (Cominig). The plates were incubated at 37°C with intermittent shaking for 20 hours. GFP fluorescence (485/528 nm; Excitation/Emission) and turbidity (600nm) were measured every 5 minutes with in a BioTek Synergy HT-I plate reader.
References


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Chapter 2

DNA damage and resulting functional interactions between *Escherichia coli* DNA Pol III and DNA Pol IV.

Abstract

High intracellular concentrations of *Escherichia coli* DNA Pol IV (DinB) effect growth arrest resulting from a DNA damage-independent replication checkpoint. The underlying mechanisms governing interactions between bacterial DNA polymerases during DNA polymerase exchange upon DNA damage treatment and/or upon activation of DNA replication checkpoints are not well understood. To gain insight into these mechanisms, we hypothesized that upon treatment with DNA damaging agents, the interactions between DinB and the replicative DNA Pol III should be identifiable *in vivo* as growth arrest at low DinB intracellular concentrations. We devised a genetic system with strains bearing a chromosomal allele encoding an antimutator DNA Pol III α-subunit (*dnaE915*) and various *dinB* alleles to study growth arrest in the presence or absence of DNA damage treatment. Thus, a *dnaE915* strain with either a chromosomal copy of a catalytically deficient variant of *dinB* (*dinB(D103N)*) or a low copy number plasmid expressing *dinB* under its own promoter, is more sensitive to DNA damage treatment than strains lacking *dinB*. Remarkably, in the absence of DNA damage treatment, a *dnaE915* strain expressing *dinB(D103N)* from the same plasmid backbone as *dinB* has both aberrant colony morphology and poor growth in liquid rich medium. The selective pressure imposed by the genetic conditions indicated above, allowed us to identify *dinB* intragenic suppressor mutations that eliminated growth defects. Mapping of these mutations uncovered a region on DinB, likely important for protein-protein interactions.
governing either polymerase exchange or checkpoint activation. The findings presented here reveal insights into the interplay between different DNA polymerases upon DNA damage.

**Introduction**

All cells accumulate DNA damage that, if left unrepaired, will stall DNA synthesis due to the inability of high fidelity DNA polymerases to use lesion-containing DNA as template [1]. DNA replication stalling is a potentially lethal event. To prevent death, cells respond to replication fork stalling by upregulating the expression of specialized low fidelity DNA polymerases, which can perform translesion synthesis (TLS) consisting of both insertion opposite to and elongation from lesion-containing templates [2]. TLS is potentially a low fidelity process resulting in elevated mutagenesis, which could in turn result in fitness acquisition, such as antibiotic resistance in bacteria [3, 4]. In fact, recent data suggests that mutations responsible for resistance to several classes of antibiotics require TLS DNA polymerases [5, 6]. Moreover, these DNA polymerases appear to play a role in the development of disorders such as cancer in metazoans [7-11]. Despite recent advances, there is still much to learn about the *in vivo* interactions between TLS and replicative DNA polymerases.

TLS DNA polymerases are known to play a role in replication checkpoints in eukaryotes [12-15]. There is mounting evidence that these DNA polymerases may also act as rudimentary checkpoints or DNA replication “brakes” in prokaryotic cells. Remarkably, these checkpoints appeared to be independent of catalytic function, and thus protein-protein interactions likely play an important role in the process [16-18]. The *in vivo* outcome of checkpoints or growth arrests has been measured in bacteria as either
cold sensitivities ([16, 19]) or poor survival upon vast overexpression of TLS DNA polymerases [17, 20].

It has been shown that the overproduction of DinB, one of the TLS DNA polymerases in *E. coli*, results in growth arrest likely due to displacement of the catalytic subunit (Pol IIIα) of the replicative DNA polymerase from replication forks [17, 21]. It has also recently been suggested that cytotoxicity is the result of high levels of DinB inserting a damaged nucleotide (8-oxo-dG) into DNA, leading to double strand DNA breaks [20]. The underlying mechanism of this growth arrest phenomenon is not well understood.

Notably, *in vitro* assays reveal DinB can displace (Pol IIIα) from the processivity clamp (β-clamp) and/or the DnaB helicase at physiological concentrations of these proteins [17, 22]. When compared to DNA Pol IIIα, DinB’s binding to the β-clamp or helicase would slow or even interrupt DNA replication [22]. However, it is unknown how a DinB-mediated replication brake would function *in vivo* at near physiological concentrations or upon DNA damage treatment. Perhaps DNA lesions themselves control a DinB-mediated brake *in vivo* by targeting DinB to the stalled replication fork.

*E.coli* strains lacking the *dinB* gene (Δ*dinB*) are sensitive to nitrofurazone (NFZ) and 4-nitroquinoline-1-oxide (4-NQO) [23], reagents that generate persistent DNA lesions on the *N*^2^ group of deoxyguanine (*N*^2^-dG) as well as alkylating agents such as methyl methanesulfonate (MMS) [24]. The introduction of a low copy number plasmid expressing *dinB*^+^ under its native promoter is able to complement the NFZ or MMS sensitivity of Δ*dinB* strains [23-25].

We were interested in learning about the underlying mechanisms governing
interactions between different bacterial DNA polymerases upon DNA damage treatment, which is likely to activate either DNA polymerase exchange and/or DNA replication checkpoints. Thus, we set up a genetic system that would permit us to detect interactions between DNA polymerases as lack of growth upon treatment with DNA damaging agents. This system is composed of strains with both an antimutator dnaE allele (dnaE915) [26], encoding a variant of Pol IIIα, and dinB+ and its alleles. Pol IIIα(915) has been hypothesized to be an antimutator because it synthesizes DNA slower than DNA Pol IIIα [26, 27] or inhibits low fidelity DNA synthesis by the TLS DNA polymerases, specifically DinB [28]. We hypothesized that regardless of the mechanism, it was likely that DNA Pol IIIα(915) would interact differently with DinB compared to DNA Pol IIIα and such interaction would result in detectable growth arrest. Indeed, this allele combination allowed us to detect DinB dependent growth arrest at in vivo DinB intracellular concentrations much lower than that of previous studies [17].

We find dnaE915 sensitizes cells to a DinB mediated growth arrest both with and without DNA damage treatment. The DNA damage-induced growth arrest is observed in dnaE915 cells carrying either a chromosomal copy of a catalytic deficient dinB allele (dinB(D103N)), or dinB+ expressed from a low copy number plasmid. Moreover, expression of DinB(D103N) from the same low copy number plasmid in a dnaE915 strain results in growth defect without DNA damage treatment. Because all of the above genetic situations result in severe growth defects, we were able to identify a variety of intragenic dinB suppressor mutations. Notably, most point mutations and small insertions were localized to a specific area of DinB. Taking these data together, we infer that this DinB face is interacting directly or indirectly with DNA Pol IIIα. This is the first
evidence identifying a region of the DinB protein functionally interacting with the replicative DNA Pol IIIα. This information is key in gaining further insights into the mechanism underlying DinB mediated processes such as DNA polymerase exchange [1, 29] or DNA replication checkpoints [17].

**Materials and Methods**

**Bacterial Strains and Plasmids.** Bacterial strains and plasmids are listed in Appendix B. Oligonucleotides used in site directed mutagenesis or other recombinant DNA techniques including plasmid construction are also listed in Appendix B. dinB alleles were introduced into the pBAD18 overexpression vector [30] using standard recombinant DNA techniques. dinB\(^+\), dinB(F292Y), or dinB(V7G) were amplified from pYG768 [31, 32] based plasmids by PCR using primers that added Xba I and Kpn I restriction sites. All other dinB alleles in pBAD18 were generated using site direct mutagenesis with the GeneTailor Site-Directed Mutagenesis System (Life Technologies) into pBAD18(dinB\(^+\)).

Methylase from New England Biolabs was used to methylate plasmids before site directed mutagenesis. Low copy number plasmid (pYG768;[31, 32])-borne DinB mutants were constructed by site-directed mutagenesis [25]. Plasmids were introduced into CaCl\(_2\) chemically competent cells by transformation [33]. Chromosomal deletions or gene alleles were moved between different *E. coli* strains via P1 transduction [33]. Mutations were verified by DNA sequencing, which was carried out at the Tufts University Core Facility in Boston, MA.

**DinB overproduction and determination of CFUs.** The different DinB variants were overproduced with arabinose from pBAD18 [30] following the protocol of Uchida et al. [17]. Exceptions are the 1X M9 salt and M9 minimal medium supplemented with
casamino acids that we used in these experiments. Cultures were split and L-arabinose (0.2%) was added to half of the culture while the other half was left uninduced to serve as control. Aliquots were removed at the times indicated in the figures, serially diluted on rich medium with 0.2% glucose and incubated at 37°C. Ampicillin (Amp, 200 µg/mL; Sigma) was added to all growth media to ensure plasmid retention. The relative levels of DinB\(^+\), DinB(F292Y), or DinB(V7G) in \(\Delta\text{dinB}\text{dnaE915}\) were determined using immunoblotting as before [34].

**Determination of survival to DNA damaging agents.** These were carried out as before [25]. The DNA damaging agents used here are methyl methanesulfonate (MMS; Acros Organics), nitrofurazone (NFZ; Sigma), ciprofloxacin (Cip; Sigma), or UV irradiation (254 nm) at the levels of treatment shown in the respective figure legends.

**Detection of suppression of synthetic sickness.** \(pdinB(D103N)\) was introduced into chemically competent \(\text{dnaE915}\) cells by transformation, spread onto LB with Amp, and incubated at 37°C. After 19-22 hrs, transformant colonies were visualized at low magnification (20-40X) with a Nikon SMZ800 microscope. To determine CFU/colony, standard procedures [33] were carried out after resuspending colonies in an isotonic solution. Strains containing plasmids with intragenic DinB suppressors, identified for their ability to grow to saturation after approximately 20 hr of incubation, were purified (Qiagen’s plasmid Miniprep Kit), and both promoter and DinB(D103N) open reading frame were sequenced. Similar procedures were carried out for \(\Delta\text{dinB}\text{dnaE915}/pdinB^+\) strains, which were hypersensitive upon MMS treatment.

**SOS Induction Assays.** Cells bearing a chromosomal copy of a sulA promoter-GFP fusion reporter (\(\text{sulA}-\text{p-GFP}; [35]\)) were grown to saturation in minimal medium with
Amp (200 µg/mL), diluted 1 to 10 in the same growth medium with MMS or ciprofloxacin (Cip; Sigma) and green fluorescent protein (GFP) expression (485/528 nm; excitation/emission) was measured in a BioTek Synergy HT-I plate reader [25]. 10,000 cells from a colony resuspended in NaCl (1%) were used to measure GFP expression in a fluorescence activated cell sorter (BD FACSaria).

**Electrophoretic mobility shift assay (EMSA).** Protein-DNA binding reactions were incubated in 20 µL of a 1X reaction buffer (500 mM HEPES pH 7.5, 1.5 M KCl) at 30°C for 35 min. 25 nM of DNA was used in all reactions with variations of 15, 35, or 70 pmol of native DinB protein. The reaction was separated by electrophoresis on a 6% native polyacrylamide gel (0.5X TBE, 0.01 µg/mL BSA) at 65 V. The gel bands were visualized on a Typhoon 8600 phosphor-imager from Amersham.

**DNA Polymerase extension assays.** Native DinB variants were purified and assayed as before [34] with 2.5 µM of DinB or its variants per reaction.

**Circular dichroism (CD) spectroscopy.** The secondary structure of DinB⁺ and DinB(F292Y) were analyzed as before [34] using a Jasco J-715 spectropolarimeter.

**Mutation Assays.** We carried out the same procedure as before [25, 36] to identify DNA damage (NFZ; 7.5 µM) induced mutants. Three independent experiments were carried out and at least 362 colonies were screened per isolate.

The frequency of rifampicin resistant (Rif®) mutants in the ΔdinBdnaE915/pdinB(D103N) strain at different stages, namely stage 1, 2, and 3 as indicated in Fig S2A was determined. Colonies from stage 1 were suspended an isotonic buffer and spread onto LB medium with rifampicin (Rif; 100 µg/mL; Calbiochem). Stages 2 and 3 colonies were grown to saturation in liquid LB medium and inoculated
onto medium containing Rif. In all cases, Rif\(^R\) colonies were counted after 24 hr of incubation at 37\(^{\circ}\)C. The number of viable cells was also determined in each experiment.

**Results**

**dnaE915 cells are more susceptible to a DinB dependent growth arrest than dnaE\(^+\)**

To study interactions of DinB with the replicative machinery, we chose a strain carrying a dnaE allele (dnaE915; [26, 27]) encoding a variant of the DNA Pol III catalytic \(\alpha\)-subunit (DNA Pol III\(\alpha\)(915)) that is thought to interact differently with DinB than wild type DNA Pol III\(\alpha\) [28]. Therefore, we introduced a plasmid borne copy of DinB\(^+\) regulated by an arabinose inducible promoter (pBAD18\(\text{dinB}^+\)) into a dnaE915 strain with a deletion of the chromosomal \(\text{dinB}\) gene (\(\Delta\text{dinBdnaE915}\)). Notably, we find these dnaE915 cells to be 100 fold more sensitive to DinB\(^+\) overexpression than dnaE\(^+\) (Fig 1).

![Figure 1. dnaE915 cells are more susceptible to DinB overproduction growth arrest than dnaE\(^+\) cells.](image)

(A) Samples were collected from strains at times indicated with (filled symbols) or without (empty symbols) arabinose induction. dnaE915 strains are more sensitive than dnaE\(^+\) to overproduction of DinB variants regardless of their catalytic activity. (B) Overproduction of DinB\(^+\) (filled triangle) but not DinB(F13V) (filled square) is toxic to \(\Delta\text{dinBdnaE915}\). Error bars represent the standard deviation of the mean from at least 3 independent experiments.
Figure 2. Chromosomal \textit{dinB(D103N)} or plasmid borne \textit{dinB} \textsuperscript{+} effect a DNA damage inducible growth arrest in \textit{dnaE915} cells. (A) \textit{dnaE915} strains with chromosomal \textit{dinB} alleles were treated with MMS (7.5 mM; gray bars) or NFZ (7.5 µM; white bars). Only those carrying \textit{dinB(D103N)} are MMS or NFZ hypersensitive. (B) \textit{ΔdinBdnaE915} and \textit{dinB(D103N)dnaE915} strains survive at similar levels upon UV treatment (∼55 J/m\textsuperscript{2}). (C) The \textit{ΔdinBdnaE915} cells carrying \textit{pdinB} \textsuperscript{+}, \textit{pdinB(F13V)}, or \textit{pdinB(Y79A)} are hypersensitive to MMS (7.5 mM) but not NFZ (7.5 µM). \textit{ΔdinBdnaE915} with \textit{pdinB(F13V)} or \textit{pdinB(Y79A)} are as hypersensitivity to NFZ as an isogenic \textit{dnaE} \textsuperscript{+} strain carrying the same plasmids [25]. The inability to interact with the β-clamp (Δβ) abolishes hypersensitivity to MMS and resistance to NFZ. (D) The \textit{ΔdinBdnaE915} cells carrying \textit{pdinB} \textsuperscript{+}, \textit{pdinB(F13V)}, or \textit{pdinB(Y79A)} are hypersensitive to UV irradiation (∼40 J/m\textsuperscript{2}). Error bars represent the standard deviation of the mean from at least 3 independent isolates.
This may be due to DinB displacement of DNA Pol IIIα(915) and the subsequent incorporation of 8-oxo-dG during DNA synthesis [20]. Notably, overexpression of a TLS deficient DinB(F13V) variant, which is unable to incorporate 8-oxo-guanine in vitro, has no effect on growth in a dnaE+ background (Fig 1B; [20]). Conversely, in dnaE915 strains, we found that overproduction of either DinB(F13V) or DinB+ is equally toxic. Similar results were obtained with either a translesion synthesis deficient derivative, dinB(Y79A), or with the catalytically inactive dinB(D103N) (Fig 1A). Curiously, the comparatively lower cellular level of DinB(D103N) expressed in uninduced cells is enough to slow cell growth (Fig 1A). Therefore the overproduction toxicity observed is independent of DinB catalytic or translesion activity and is perhaps dependent on direct or indirect interactions between DinB and DNA Pol IIIα(915).

**dnaE915 strains with a chromosomal copy of dinB(D103N) are hypersensitive to MMS or NFZ treatment.**

dnaE+ ΔdinB strains expressing plasmid borne DinB+ with a mutation in the catalytic D103 residue (pdinB(D103N)) are hypersensitive to both NFZ and MMS, i.e. more sensitive to treatment than a ΔdinB isogenic strain carrying only the vector [23, 25, 37]. However, we have shown that an isogenic strain carrying a chromosomal copy of dinB(D103N) is not longer hypersensitive [25]. Because the dnaE915 strain is more sensitive to DinB overexpression, we tested whether the chromosomal dinB(D103N) allele would give rise to NFZ or MMS hypersensitivity in the dnaE915 background. This allele was introduced by transduction into cells with chromosomal copies of dinB(D103N) or the TLS deficient dinB(F13V) and dinB(Y79A) alleles to assess their ability to survive NFZ or MMS treatment. Surprisingly, the dinB(D103N), dnaE915
strain is more sensitive to both NFZ and MMS (Fig 2A) when compared to the isogenic \( \Delta dinB \ dnaE915 \) strain. The DNA damage dependent hypersensitivities seen in cells bearing the chromosomal \( dinB(F13V) \) or \( dinB(Y79A) \) TLS deficient alleles [25] are likely due to the toxic consequences of incomplete TLS [37], which we show here is independent of the \( dnaE915 \) allele (Fig 2A). \( \Delta dinBdnaE915 \) and \( dinB(D103N)dnaE915 \) survive equally well upon UV irradiation (Fig 2B), a treatment which generates lesions that DinB is unable to bypass, demonstrating hypersensitivity is lesion specific.

**Figure 3. DinB prefers lesion-containing DNA.** (A) Schematic of a primer template junction containing an adenine or 3-deaza-3-methyl-adenine (3-dMeA). The HEX fluorescent tag is depicted as a star. (B) An electrophoretic mobility shift assay (EMSA) reveals DinB\(^+\) prefers lesion-containing DNA better than undamaged DNA. 70 pmol DinB\(^+\) and 0.025 pmol DNA are shown. (C) The ratio of shifted to unshifted DNA upon addition of the indicated amounts of DinB is shown. The graph was created with Image J software.
In an attempt to understand the hypersensitive phenotype we conducted a gel shift assay to compare the relative affinity of DinB\(^+\) for a template containing an undamaged adenine or a 3-meA analogue (3-deaza-3-methyl-adenine). We find that DinB\(^+\) has preference for lesion-containing DNA relative to undamaged DNA (Fig 3), suggesting that this might be the reason, or at least part of it, to explain the observed *in vivo* growth arrest.

**DNA damage is also required for pdinB\(^+\) -dependent growth arrest in dnaE915 cells**

We sought to slightly increase the intracellular levels of native DinB by expressing it from its own DNA damage inducible promoter on a low copy plasmid [31]. We reasoned we would observe growth defects in ΔdinBdnaE915 cells with the combination of an increase in copy number and DNA damage treatment to help target DinB to the stalled replication fork. Therefore, we treated a ΔdinB,dnaE915/pdinB\(^+\) strain with MMS. The pdinB\(^+\) plasmid has been found to express DinB at 5-10 times higher levels (i.e. between 1250-2500 molecules/cell at uninduced levels or 10 times more molecules/cell upon SOS induction) than the chromosome [31, 32]. These levels are lower than the *in vivo* assays using overexpression vectors such as Fig 1, which have an estimated level of DinB that is 15-72 times the intracellular concentration of a fully SOS induced cell (i.e. 30,000-144,000 molecules/cell) [17].

Indeed, we find that expression of plasmid borne *dinB\(^+\)* results in hypersensitivity to MMS (Fig 2C). We also find that cells carrying plasmid borne *dinB*(F13V) or *dinB*(Y79A) are as hypersensitive to MMS as cells carrying pdinB\(^+\) (Fig 2C). As with the chromosomal alleles, these strains are hypersensitive to NFZ whether they are dnaE\(^+\) [25] or dnaE915 (Fig 2D). The results suggest DinB likely interacts differently with DNA Pol
IIIα(915) upon MMS treatment, and the hypersensitive-dependent phenotype of TLS deficient variants is independent of interactions with DNA Pol IIIα(915). To exclude the possibility our findings are unique to the P90C strain, we recreated this phenotype in a wild type *E. coli* MG1655 strain. The *dnaE915* allele and *pdinB* were introduced into a ΔdinB MG1655 strain and we find these cells to be also hypersensitive to MMS (Fig 4).

It was possible we observed a robust DinB-mediated hypersensitivity in ΔdinB*dnaE915*/pdinB cells upon treatment with MMS but not NFZ (Fig 2C) because MMS is a stronger inducer of the SOS gene network than NFZ [25]. Therefore, we irradiated cells with ultraviolet radiation (UV) or treated them with Ciprofloxacin (Cip; 0.16 µg/mL), both potent inducers of SOS gene network [1, 25]. We find that ΔdinB*dnaE915* expressing either pdinB, pdinB(F13V), or pdinB(Y79A) are hypersensitive to UV (Fig 2D) and Cip (data not shown); indicating intracellular levels of DinB (and the TLS deficient variants), are likely responsible for the observed growth arrest phenotype. There is no dependence on SOS induction for the growth arrest observed in the *dinB(D103N)dnaE915* strain, which is hypersensitive to both NFZ and
MMS treatments but not UV (Figs 2A and B). Interestingly, strains expressing DinB(F13V) can partially suppress UV or Cip hypersensitivities but not sensitivity to an agent that forms DinB cognate lesions (MMS). It is possible this rescue is related to the suppression of the DinB overexpression growth arrest in dnaE+ strains by the F13V mutation (Fig 1; [20]).

Thus, it seems that cells undergo a DinB+ dependent growth arrest that could be triggered by a variety of conditions that generate replication stress and genomic instability, provided the intracellular local concentration of native DinB+ is high enough. Moreover, DinB cognate lesions do seem to play a specific role in this growth arrest phenotype, perhaps through DNA polymerase targeting.

To determine if the interactions that we observe are the product of events occurring at the replication fork, a plasmid carrying a dinB allele without the β-clamp binding motif (dinBΔβ) was constructed and introduced by transformation into dnaE915 cells. These cells survive MMS treatment at levels identical to cells carrying the vector (Fig 2C). This evidence suggests that the interactions must be happening at the replication fork, consistent with the idea of an increase in the DinB+ local concentration.

**Genetic interactions between dnaE915 and pdinB(D103N) result in synthetic sickness due to replication stress.**

Because the dnaE915 strain with the chromosomal dinB(D103N) allele is hypersensitive to DNA damage treatment, we chose to study it further. Moreover, any survival effects observed with this allele are independent of enzymatic activity, and likely dependent on protein-protein interactions. Unexpectedly, when we introduced a low copy number plasmid with dinB(D103N) expressed from its native promoter (pdinB(D103N);
[31]) into the ΔdinBdnaE915 strain, we find an aberrant colony morphology in the absence of DNA damage treatment when compared to isogenic strains with either the vector or pdinB+ (Fig 5).

Synthetic sickness generated by genetic interactions between dnaE915 and plasmid borne dinB(D103N) results in colonies that are translucent and flat (Fig 5A) with ~10 fold less CFU/colony compared to colonies formed by isogenic strains containing only the vector of approximately the same diameter (Fig 5B). Subculturing these colonies in liquid LB medium led to non-saturated cultures after 20 hr at 37°C (Fig 5C). Curiously, subsequent cultures generated both full saturation and formation of normal looking colonies on LB medium (Fig 6).

Figure 5. The combination of dnaE915 and pdinB(D103N) alleles results in synthetic sickness with no DNA damage treatment. Colonies formed by ΔdinBdnaE915 pdinB(D103N) have altered morphology and contain fewer cells. (A) Representative colonies of ΔdinBdnaE915 with pdinB(D103N) or pVector at 20X. (B) ΔdinBdnaE915 pdinB(D103N) colonies have fewer cells than dinBdnaE915 carrying pVector. (C) Cultures of ΔdinBdnaE915 pdinB(D103N) in LB rich medium do not reach saturation after 24 hrs and have ~1000 fold less CFUs/mL than ΔdinBdnaE915 carrying pVector or other plasmid borne DinB variants. Error bars represent the standard deviation of the mean from at least 3 independent experiments.
These growth defect phenotypes are absent when the same strain expresses the plasmid borne \( \text{dinB}(D103N) \) lacking the \( \beta \)-clamp (\( \text{pdinB}(D103N)\Delta\beta \); Fig 5C). The requirement of DinB’s \( \beta \)-clamp binding motif to observe the growth defects described here and above (section 3.3) suggest that DinB(D103N) (and DinB\(^+\)) need to be localized at the replication fork to cause hypersensitivity. Once there, the catalytically inactive

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**Figure 6. Schematics of the generation of mutations that suppress DinB dependent growth defects.** (A) Generation of suppression mutations to rescue growth defects in the \( \Delta\text{dinBdnaE915} \ \text{pdinB}(D103N) \) strain. Stage 1 is the initial synthetic sick \( \Delta\text{dinBdnaE915} \ \text{pdinB}(D103N) \) transformants. Stage 2 is the subculture started from the synthetic sick colonies that fail to reach saturation after 20 hrs at 37°C. Stage 3 is the subsequent LB liquid cultures with suppressor mutations that allow cells to reach saturation and form colonies with normal morphology. (B) Generation of mutations that suppress DinB dependent MMS hypersensitivity in \( \text{dnaE915} \). \( \Delta\text{dinBdnaE915} \ \text{pdinB}^+ \) cells are first treated with MMS. Surviving colonies are then grown in LB liquid medium to saturation. The cells are plated back onto LB medium with MMS to determine presence of MMS hypersensitivity. See results and materials and methods sections for additional details.
DNA polymerase may stall DNA replication. If that were the case, the SOS gene network should be induced [1]. Therefore green fluorescent protein (GFP) under an SOS inducible promoter (sulAp) was introduced by transduction into ΔdinBdnaE915 and used as an indicator of SOS induction. Thus, GFP expression will be detected uniquely in cells when replication stalling is taking place. The vector, pdinB+ or pdinB(D103N) were introduced into ΔdinBdnaE915sulAp-GFP by transformation and SOS induction was measured using a fluorescent activated cell sorter (FACS) to identify the fraction of fluorescent cells in the population.

Figure 7. Antagonistic genetic interactions between dnaE915 and plasmid borne dinB(D103N) induce the SOS gene network with no DNA damage treatment. SOS induction of cells within colonies was determined using a fluorescence activated cell sorter (FACS). ~25% of cells in ΔdinBdnaE915sulAp-GFP pdinB(D103N) colonies with the synthetic sick morphology undergo SOS induction. A small population (4%) of ΔdinBdnaE915sulAp-GFP pdinB+ cells is SOS induced. There is negligible SOS induction in ΔdinBdnaE915 sulAp-GFP pVector or ΔdinBdnaE+ sulAp-GFP with any DinB variant tested. The measurements are the result 10,000 cells from 3 independent colonies of each strain used.
A substantial portion of the population of ΔdinBdnaE915/pdinB(D103N) cells from a resuspended colony (~25%) is fluorescent when compared to a colony from the isogenic strain with the vector alone (1%) (Fig 7). A smaller fraction of ΔdinBdnaE915/pdinB+ cells (4%) also undergo SOS induction in the absence of DNA damage indicating that DinB+ alone effects some level of replication stalling (Fig 7). There is no measurable SOS induction in ΔdinBdnaE+ strains carrying any plasmid borne allele of dinB (Fig 7). Therefore replication stress arises from interactions between DinB(D103N) (or to a lesser degree DinB+) and the DNA Pol IIIα(915) variant.

To determine if the observed synthetic sickness phenotype was happening because cells were replicating too fast in rich medium, we deposited ΔdinBdnaE915/pdinB(D103N) onto minimal medium agar plates immediately after the plasmid was introduced by transformation. We predicted that the slower growth rate ensured by minimal medium would eliminate the synthetic sickness because cells would have more time to resolve replication stress. Surprisingly, this is not the case and we find no colonies on these plates. Thus, these data indicate that the combination of the dnaE915 and pdinB(D103N) trigger a pathway that leads to cell death on minimal medium.

The pdinB(D103N) was also introduced into ΔdinBdnaE915 MGI655 cells. We find the aforementioned antagonistic genetic interaction between dnaE915 and plasmid borne dinB(D103N), again suggesting our findings are strain independent (Fig 4).

The dnaE915/pdinB(D103N) growth defect is independent of the expression of other SOS genes

To assess if other SOS induced gene products are required for the synthetic sickness observed in ΔdinBdnaE915/pdinB(D103N), we constructed an isogenic strain
with a recA deletion (ΔrecAΔdinBdnaE915), a gene product required for SOS induction. If the growth arrest phenotype were abolished in this strain, we would conclude that other SOS genes are involved in the phenomenon. However, like the isogenic recA+ strain (Fig 5), the colonies of ΔrecAΔdinBdnaE915/pdinB(D103N) are flat, translucent, and contain ~10 fold less CFU/colonies than colonies formed by isogenic strains with the vector (data not shown). Furthermore, transformant colonies grown overnight in liquid LB do not reach saturation in 20 hr and the cultures contain ~10 fold less CFU/mL than the isogenic strain with the vector (Fig 8).

Interestingly, though the synthetic sickness is evident in ΔrecA strains, these cells fail to acquire the full growth and normal colony morphology upon subsequent culturing as in the recA+ strain (Fig 6B). This indicates that, although other SOS genes are not required for synthetic sickness, they are likely involved.

**Figure 8. Synthetic sickness in ΔdinBdnaE915 pdinB(D103N) cells is independent of SOS gene network induction.** Saturated cultures of SOS deficient ΔdinBdnaE915 pdinB(D103N) colonies grown in LB rich medium have ~10-100 fold less CFUs than isogenic strains carrying pVector. Error bars represent the standard deviation of the mean from at least 3 independent isolates.
in generating mutations that suppress this phenotype. To test whether this is due to the lack of RecA itself, we constructed isogenic recA\(^{+}\) strains that are SOS deficient. Similar phenotypes are found in recA\(^{+}\) dnaE915 strains that are unable to induce the SOS response because of an uncleavable form of the SOS repressor lexA (lexA3) \([1]\) or due to a deletion of the RecQ helicase gene (\(\Delta\text{recQ}\)) \([38]\) (Figs 8 and Fig 9). Remarkably, in these SOS deficient strains, we again observed no suppression of growth defects upon subculturing for two consecutive times. Thus, it seems that RecA’s role in the DinB(D103N)-mediated growth defect phenotype is due to its ability induce the SOS gene network. It also appears that SOS induction is responsible for the robustness of the synthetic sickness, and suppression of growth arrest. We assessed the SOS induction in these cells and find that both \(\Delta\text{recA}\) and lexA3 are indeed deficient to induce the SOS response (Fig 9). We also determined that there is an intermediate level of SOS induction in \(\Delta\text{recQ}\Delta\text{dinBdnaE915}/\text{pdinB(D103N)}\). This might be the reason to explain the ~10-fold fewer CFU/mL in a liquid culture than isogenic \(\Delta\text{recA}\) or lexA3 strains (Fig 8).

We also wanted to determine how the synthetic sickness phenotype may change if the SOS genes were fully induced. This was tested by attempting to introduce the \(\text{pdinB(D103N)}\) by transformation into the \(\Delta\text{dinBdnaE915}\) strain with a lexA(Def) allele which permits constitutive expression of the SOS gene network. The genetic interaction between dnaE915 and plasmid borne dinB(D103N) results in synthetic lethality in lexA(Def) strains. This result suggests again that elevating the intracellular concentration of this DinB derivative enhances the growth defect.

The dnaE915 allele reduces DNA damage induced mutations that are dependent on a TLS deficient dinB allele.
Because it has been proposed that *dnaE915* is an antimutator due to the exclusion of DinB variants from the replication fork by DNA Pol IIIα(915) [28], we reasoned the *dnaE915* allele would reduce the frequency of NFZ-induced mutations that are dependent on *pdinB*(Y79A). As predicted, we find the Δ*dinB*Δ*dnaE915/pdinB*(Y79A) strain has a marked decrease (~60%, from 1%±0.05% SEM [25] to 0.3%±0.005% SEM) in NFZ-induced mutant frequency when compared to the Δ*dinB*Δ*dnaE*+/pdinB*(Y79A) strain. The reduction of DinB(Y79A) dependent DNA damage induced mutations suggests that perhaps *dnaE915* does result in exclusion of DinB(Y79A) from the replisome. However, *dnaE915* does not completely abolish the *pdinB*(Y79A) mutator phenotype as there are still ~23% more mutants upon NFZ treatment than Δ*dinB*Δ*dnaE*+/pVector (0.07%±0.05% SEM [25]), suggesting DinB(Y79A) is not completely excluded from the replication fork by DNA Pol IIIα (915). As seen before [25], there is a lack of a strict correlation between enhanced sensitivity and increased mutagenesis.

**The rapid accumulation of mutations suppress both synthetic sickness and DNA damage treatment dependent growth arrest.**

As stated above, although the Δ*dinB*Δ*dnaE915/pdinB*(D103N) strain does form colonies with aberrant morphology and does not grow to saturation in LB medium, cells regain normal growth upon further culturing (Fig 6A). We surmised this robust growth was the product of suppressor mutations selected as the result of genetic interactions between the *dnaE915* and *pdinB*(D103N) alleles. Similarly, we also hypothesized the suppression of the MMS hypersensitivity in Δ*dinB*Δ*dnaE915*Δ*sulAp*-GFP cells with *pdinB*+ was also due to mutations. Nine colonies that formed during MMS treatment were subcultured in LB, grown to saturation and treated with MMS (Fig 6B).
We find all isolates lose their hypersensitivity to MMS treatment (Figs 10A and 11). In fact, several of the isolates now display resistance to MMS similar to that found in $\Delta$dinB$^{+}$/p$\text{dinB}^{+}$ [25]. Nine additional $\Delta$dinB$^{+}$/p$\text{dinB}^{+}$ isolates were treated as before (Fig 6B), but with a higher amount of MMS (9 mM) and we again find that none retain hypersensitivity to MMS (data not shown). Interestingly, all isolates, other than the one carrying the plasmid with a thirteen-basepair deletion in the $\text{dinB}$ open reading frame, survive NFZ treatment better than cells carrying the vector (Fig 10A and 11), suggesting the DinB expressed in these cells has retained total or partial TLS activity. Furthermore, we find all isolates induce $\text{sulA}$ upon MMS or Cip treatment (Fig 12) and thus are SOS

Figure 10. **Suppressor mutations are required for dnaE915 pdinB$^{+}$ cells to become MMS resistant.** (A) $\Delta$dinBdnaE915 pdinB$^{+}$ cultures started from colonies pretreated with MMS (7.5 mM) were treated with NFZ (7.5 µM: white bars) or MMS (7.5 mM: gray bars) for a second time. All cultures lose MMS hypersensitivity. pdinB$^{+}$ MMS I is a MMS treated $\Delta$dinBdnaE915 pdinB$^{+}$ representative strain without a mutation in $\text{dinB}$ or its promoter. Error bars represent the standard deviation of the mean from at least 3 independent experiments. (B) Mutations in plasmid borne $\text{dinB}$ are required for MMS hypersensitivity suppression upon their reintroduction into $\Delta$dinBdnaE915. Error bars represent the standard deviation of the mean from 3 independent isolates.

We find all isolates lose their hypersensitivity to MMS treatment (Figs 10A and 11). In fact, several of the isolates now display resistance to MMS similar to that found in $\Delta$dinB$^{+}$/p$\text{dinB}^{+}$ [25]. Nine additional $\Delta$dinB$^{+}$/p$\text{dinB}^{+}$ isolates were treated as before (Fig 6B), but with a higher amount of MMS (9 mM) and we again find that none retain hypersensitivity to MMS (data not shown). Interestingly, all isolates, other than the one carrying the plasmid with a thirteen-basepair deletion in the $\text{dinB}$ open reading frame, survive NFZ treatment better than cells carrying the vector (Fig 10A and 11), suggesting the DinB expressed in these cells has retained total or partial TLS activity. Furthermore, we find all isolates induce $\text{sulA}$ upon MMS or Cip treatment (Fig 12) and thus are SOS
proficient. Therefore, the suppression of MMS hypersensitivity is not the result of a reduction in the SOS response.

To test if the putative suppressor mutations were located on the plasmid, we introduced pdinB+ plasmids isolated from MMS-treated ΔdinBdnaE915 back into plasmid-free isogenic cells. If the suppressor mutations are located on the plasmid, the resulting transformants will lack MMS hypersensitivity. We find only some of these plasmids abolish MMS sensitivity (Fig 10B), indicating both intragenic and extragenic suppressor mutations eliminate DinB dependent MMS hypersensitivity in dnaE915 cells.

To identify the suppressor mutations, we purified plasmids from thirty isolates of ΔdinBdnaE915/pdinB(D103N) that have acquired full growth. pdinB+ from eighteen...
isolates that are no longer hypersensitive to MMS were also purified. Both the promoter and the \(dinB(D103N)\) or \(dinB^+\) coding regions of these plasmids were sequenced. In this fashion, we identified various intragenic suppressor mutations in \(p\ dinB(D103N)\) or \(p\ dinB^+\). The mutations we find in \(p\ dinB(D103N)\) include point mutations, duplications, large rearrangements, deletions, and even an insertion sequence (\(\text{insB}\)) (Fig 13A). Of the nine bp substitutions found there are two transition (T to C) and 7 transversions (four T to A; two T to G, and one A to C). As expected, we find basepair substitutions in (L349P; [39]) or near (L307Q; [40]) residues of DinB that are important for interactions with the \(\beta\)-clamp. We had already identified DinB’s \(\beta\)-clamp binding motif as an area of the protein that is required for synthetic sickness (Fig 5C). Two mutations, a bp substitution (V288G) and an in frame duplication of three amino acids

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**Figure 12.** Suppression of MMS hypersensitivity in \(\Delta dinBdnaE915\) \(pdinB^+\) cells is not due to the inability to induce the SOS gene network. SOS induction is unchanged in MMS pretreated \(\Delta dinBdnaE915\) \(pdinB^+\) cells. Representative fluorescence kinetic curves of \(\Delta dinBdnaE915\) sulAp-GFP \(pdinB^+\) cells with MMS hypersensitivity suppressor mutations. Cells were treated in duplicate with MMS (7.5 and 10 mM) or Cip (0.1 µg/mL) to induce SOS gene network.

\(dnaE915\) isolates that are no longer hypersensitive to MMS were also purified. Both the promoter and the \(dinB(D103N)\) or \(dinB^+\) coding regions of these plasmids were sequenced. In this fashion, we identified various intragenic suppressor mutations in \(pdinB(D103N)\) or \(pdinB^+\). The mutations we find in \(pdinB(D103N)\) include point mutations, duplications, large rearrangements, deletions, and even an insertion sequence (\(\text{insB}\)) (Fig 13A). Of the nine bp substitutions found there are two transition (T to C) and 7 transversions (four T to A; two T to G, and one A to C). As expected, we find basepair substitutions in (L349P; [39]) or near (L307Q; [40]) residues of DinB that are important for interactions with the \(\beta\)-clamp. We had already identified DinB’s \(\beta\)-clamp binding motif as an area of the protein that is required for synthetic sickness (Fig 5C). Two mutations, a bp substitution (V288G) and an in frame duplication of three amino acids
occurred at least two times in independent isolates. We find three of the eighteen plasmids had mutations in the \textit{dinB}^+ gene (one with a deletion of the gene between 353 bp and 366 bp) and two with transversion mutations in different areas of the gene (F292Y;T to A or V7G;T to G) (Fig 10). As predicted, these point mutations occurred in the plasmids that do not cause MMS hypersensitivity upon retransformation (Fig 10B).

Many of the mutations are localized to a specific area of the protein; in fact the V288 and F292 residues are part of the same beta sheet (Fig 13B). This area of the protein could be important for protein stability or an interface for interactions with other proteins involved in replication, including DNA Pol III\alpha.

We hypothesized that the other TLS DNA polymerases, DNA Pol II or DNA Pol V, were responsible for the mutations due to their low fidelity compared to DNA Pol’s I or III. Additionally these genes are part of the SOS gene network and, as stated above, SOS induction is required for suppression of \textit{pdinB(D103N)} dependent synthetic sickness. Thus, we constructed a \textit{dnaE915} strain with chromosomal deletions for all three TLS DNA polymerases (\textit{\Delta TLSdnaE915}), reasoning this would reduce or eliminate bp substitutions, and then introduced \textit{pdinB(D103N)}. This strain has identical growth defects as \textit{\Delta dinBdnaE915/pdinB(D103N)} (Fig 5) that disappear with subsequent culturing (Fig 6A). Interestingly, we find bp substitutions (Fig 13) in the absence of TLS DNA polymerases (one transversion of T to G and two transitions of C to T). Furthermore, the T to G transversion results in yet another independent generation of the V288G mutation. This evidence suggests that either of the canonical high fidelity DNA polymerases, DNA Pol I or DNA Pol III\alpha(915), must be responsible for the bp substitutions found in
It is plausible that in this instance of strong selective pressure the cell favors mutagenesis and allows traditionally high fidelity DNA polymerases to become error prone. Alternatively, bacterial TLS DNA polymerases might play a role in targeting various proteins to the replication fork that could aid in high fidelity DNA synthesis.

#### Table

<table>
<thead>
<tr>
<th>Mutation Class</th>
<th>Frequency in ΔdinB (%)</th>
<th>Frequency in ΔdinB(D103N) (%)</th>
<th>Frequency in ΔTLS (%)</th>
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<td>No mutation</td>
<td>83</td>
<td>50</td>
<td>11</td>
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<tr>
<td>Substitution mutation</td>
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<td>Rearrangement</td>
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<tr>
<td>Other</td>
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<td>6</td>
<td>11</td>
</tr>
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**Figure 13. A diverse array of mutations in plasmid borne dinB+ or dinB(D103N) alleles suppress growth defects.**

(A) Percentage of each mutation class in the open reading frame of plasmid borne dinB+ or dinB(D103N). (B) Mutations occur throughout the protein, however, they are mostly localized to a specific area of DinB. Only the mutated residues mentioned in this manuscript (V7, V288, F292, L307, L349, or the RGG duplication) are shown as spheres. The DinB structure is from an in silico model generated in collaboration with A. Abyzov and V. Ilyin [47]. Rendering was done with PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA.).

ΔTLSdnaE915/pdinB(D103N) cells. It is plausible that in this instance of strong selective pressure the cell favors mutagenesis and allows traditionally high fidelity DNA polymerases to become error prone. Alternatively, bacterial TLS DNA polymerases might play a role in targeting various proteins to the replication fork that could aid in high fidelity DNA synthesis.
fidelity tolerance of replication stalling, as has been seen in eukaryotes [41, 42].

We predicted V288G or \(324_{RGG326}\) mutations are particularly disruptive to the structural or functional role of DinB(D103N) in synthetic sickness because they were isolated multiple times. In turn, these mutations should suppress other \(\text{dinB(D103N)}\) sensitivity phenotypes such as the hypersensitivity to NFZ or MMS in \(\text{dnaE}^+\) cells [25]. The plasmids bearing the D103N mutation in addition to the basepair substitution \(\text{dinB}(V288G)\) or duplication \(\text{dinB}(324_{RGG326})\) were introduced into \(\Delta\text{dinB}\text{dnaE}^+\) cells. The mutations indeed suppress the \(\text{pdinB}(\text{D103N})\) mediated hypersensitivity to NFZ or MMS (data not shown) demonstrating the mutations abolish the ability of DinB(D103N) to effect DNA replication stalling/blockage in \(\text{dnaE}^+\) cells.

If the observed DinB dependent growth defects are part of similar pathways (both those found with DinB\(^+\) or DinB(D103N)), then suppressors arising in response to one condition should suppress others. Therefore DinB(V288G), (F292Y), or (V7G) should suppress any growth arrest phenotype. These base pair substitutions were reintroduced into the original \(\text{pdinB}(\text{D103N})\) or \(\text{pdinB}^+\) plasmids using site directed mutagenesis to rule out other changes that may be present elsewhere in the plasmid. \(\text{dnaE915}\) cells bearing \(\text{pdinB}(\text{D103N})\) with V288G, F292Y, or V7G form colonies with normal morphology on LB solid medium (Fig 14), indicating any of the three mutations in \(\text{dinB}(\text{D103N})\) is enough to suppress the synthetic sickness.

To further understand how these mutations affect the structure and/or function of DinB we studied the \textit{in vivo} and \textit{in vitro} TLS activity of DinB(V288G), DinB(F292Y) or DinB(V7G). We define \textit{in vivo} TLS proficiency as the ability of a strain encoding one of these DinB variants to survive NFZ or MMS treatment compared to the isogenic strain
with the vector. ΔdinBdnaE915 or ΔdinBdnaE1 strains expressing the dinB alleles were treated with NFZ or MMS. ΔdinB strains, independent of the dnaE allele, expressing the plasmid borne dinB(V288G) do not survive better than those with the vector alone (data not shown), suggesting that V288G might render DinB non-functional.

Unlike DinB(V288G), DinB(F292Y) and DinB(V7G) appear to have in vivo TLS activity. The ΔdinBdnaE915 strain carrying either pdinB(F292Y) or pdinB(V7G) are as resistant as cells carrying pdinB1 at 7.5 μM NFZ and more resistant at higher levels of NFZ (≥10 μM) (Fig 15). However, although both variants abolish cellular

Figure 14. Suppressor mutations abolish a variety of DinB dependent growth defects. (A) F292Y, V7G, or V288G mutations suppress dinB(D103N) synthetic sickness in untreated ΔdinBdnaE915 cells. Representative ΔdinBdnaE915 colonies carrying the indicated plasmids at 30X magnification. The F292Y, V7G, or V288G mutations suppress the DinB(D103N) dependent synthetic sickness. (B) A temperature sensitive dnaE486 allele is also susceptible to a pdinB1 growth arrest. The DinB1 dependent sensitivity to the semi-permissive temperature (37°C) is abolished by the F292Y or V7G mutations. Error bars represent the standard deviation of the mean from at least 3 independent isolates.
hypersensitivity to MMS, only ΔdinBΔdnaE915 cells with dinB(V7G) are resistant to MMS treatment when compared to cells carrying the vector. Both DinB variants were also introduced into ΔdinBΔdnaE915 and assayed for both NFZ and MMS resistance to assess if they might be interacting differently with DNA Pol IIIα or DNA Pol IIIα(915). ΔdinBΔdnaE915 with dinB(F292Y) or dinB(V7G) are NFZ or MMS resistant, but the levels of resistance differ from the isogenic ΔdinBΔdnaE915 strain (Fig 15). Thus, although DinB(F292Y) expression abolishes MMS hypersensitivity in dnaE915, it only confers MMS resistance in dnaE915, while DinB(V7G) expression confers greater resistance to high levels of NFZ (≥10 µM) in dnaE915 than in dnaE915.

Taking all these data together we have learned that the dnaE915 strains carrying either variant survive NFZ or MMS better than strains carrying DinB+. However, this is reversed in the dnaE+ strain where DinB+ confers similar or increased NFZ and MMS resistance when compared to DinB(F292Y) or DinB(V7G). Perhaps these variants interact different with replicative proteins or with the lesions themselves. This could change local concentration of DinB(F292Y) or DinB(V7G) at the replication fork to levels permitting lesion bypass but not affect growth arrest in dnaE915.

We also purified DinB(F292Y) and assessed its in vitro TLS activity. This derivative is expressed well and is easily purified, contrary to others described here (e.g. DinB(V7G)). We assayed the in vitro activity with undamaged or 3-meA analogue containing templates. This DinB variant appears to be equally active on undamaged and lesion containing templates (Fig 15C) supporting our in vivo findings that suggest it retains TLS activity (Figs 15A and B).
Figure 15. *dinB(F292Y)* and *dinB(V7G)* retain catalytic and lesion bypass activity.

(A) Δ*dinB dnaE915*/*dnaE*+ cells with plasmid borne *dinB*, *dinB(F292Y)* or *dinB(V7G)* were treated with NFZ to determine *in vivo* TLS activity. Cells carrying *dinB(F292Y)* (filled square) or *dinB(V7G)* (filled circle) survive better than cells with the vector (open triangle). (B) Same as (A) but with MMS. In *dnaE*+ cells, both *pdinB(F292Y)* or *pdinB(V7G)* are resistant to treatment but in *dnaE915* cells this is only observed upon expression of *pdinB(V7G)*. Δ*dinBdnaE915* pdinB(F292Y) survive MMS treatment as cells carrying vector. Thus, the degree of survival is dependent on *dnaE* allele and DNA damage treatment. Error bars represent the standard deviation of the mean from at least 3 independent isolates. (C) Standing start DNA extension assays with 25 nM of undamaged or lesion containing templates (same as those shown in Fig 3A). DNA Pol I can only synthesize DNA on undamaged templates. DinB(F292Y) or DinB+ can insert a nucleotide opposite to and extend from an adenine (A) or 3-deaza-3-methyl-adenine (3-dMeA).
Figure 16. Both F292Y and V7G suppress DinB\textsuperscript{+} overexpression growth arrest in dnaE\textsuperscript{-} or dnaE915 cells. (A) Samples were collected from strains at times indicated with (filled symbols) or without (empty symbols) arabinose induction. The F292Y (squares) and V7G (circles) mutations suppress sensitivity to DinB (triangles) overexpression in both dnaE915 and dnaE\textsuperscript{-} strains. Error bars represent the standard deviation of the mean from at least 3 independent experiments. (B) Differences in survival between strains overexpressing DinB\textsuperscript{+}, DinB(F292Y) or DinB(V7G) is independent of protein levels. Western blotting as described in Materials and Methods was used to assess whether DinB and DinB(F292Y) or DinB(V7G) are equally translated in the assay. Negligible levels of DinB variants were present at 135 min in uninduced cells. DinB(F292Y) or DinB(V7G) are detected at levels similar to DinB\textsuperscript{+} at 135 min post arabinose induction. Purified DinB\textsuperscript{+} protein (750 ng) was used for sizing purposes. Cross-reactive bands serve as protein loading controls for each lane.
The suppression of both the synthetic sickness phenotype and *dinB*+ induced MMS hypersensitivity by all three mutations (V288G, F292Y, or V7G) strongly suggests that MMS hypersensitivity and growth arrest in the absence of DNA damage are part of a similar underlying mechanism and these residues are important to effect the phenotypes. Because the F292Y and V7G mutations suppressed the DinB mediated MMS sensitivity in the *dnaE915* strain, we determined if these mutations would suppress the growth arrest associated with the overproduction of DinB. We constructed pBAD18 arabinose inducible plasmids bearing either *dinB(F292Y)* or *dinB(V7G)* and introduced them into both Δ*dinB* dnaE+ and Δ*dinB* dnaE915. We find both variants suppress the DinB overproduction growth arrest in the Δ*dinB* dnaE+ strain (Fig 16A). In fact, cells overexpressing DinB(V7G) proliferate similarly to the uninduced cells (Fig 16A). Similar trends were obtained in the Δ*dinB* dnaE915 strain with DinB(F292Y) or DinB(V7G) (Fig 16A). These two derivatives, DinB(F292Y) and DinB(V7G), are detected by immunoblot at levels similar to DinB+ in Δ*dinB* dnaE915 (Fig 16B). Thus, suppression of DinB toxicity is not due to variations in protein levels.

Because the F292Y mutation does not appear to affect protein expression or translation, we used circular dichroism to determine whether the F292Y mutation affected the overall structure of the protein. The change from the nonpolar F to the polar Y does not alter the DinB secondary structure (Fig 17). Because they are both part of the same β-sheet, we attempted to similarly test how the V288G mutation may change the protein structure. Unfortunately, both DinB(V288G) and DinB(D103N)(V288G) are insoluble. This *in vitro* data fits our hypothesis that DinB(V288G) might have a different conformation or a shorter half life than native DinB or DinB(D103N).
We also tested whether DinB-dependent hypersensitivity is observed upon DNA replication stalling that is lesion independent.

Plasmid borne $dinB^+$, $dinB(F292Y)$ or $dinB(V7G)$ were introduced into cells carrying a temperature sensitive allele of $dnaE$ ($dnaE486$) [43]. $dnaE486$ cells at the semipermissive temperature of 37°C grow much like parental cells, however, there is low-level SOS induction [38]. In $\Delta dinBdnaE486/pdinB^+$, we observed the expected growth arrest at 37°C measured as a reduction in CFUs (Fig 14B).

Again we find the F292Y or V7G mutations suppress DinB dependent growth defects and the growth of cells carrying pdinB($F292Y$) or pdinB($V7G$) is indistinguishable from those carrying the vector (Fig 14B).

**Synthetic sickness suppressor mutations are not due to a general mutator state**

SOS induction (Fig 5) and the prevalence of suppressor mutations in $\Delta dinBdnaE915/pdinB(D103N)$ cells (Fig 13A) suggest a hypermutator state. The
survivors of such a hypermutator state would bear mutations not only in the dinB gene but also randomly throughout the genome. Therefore, we measured the frequency of spontaneous Rifampicin resistant mutants (Rif\(^R\)) in the \(\Delta\text{dinBdnaE915/pdinB(D103N)}\) strain at three stages of their progression from sick to wild type-like phenotypes (Fig 6A). We considered the first stage to be a colony from the transformation experiment in which the pdinB(D103N) plasmid is introduced into the \(\Delta\text{dinBdnaE915}\) strain (Fig 5). The second stage was isolated from cells that fail to reach saturation in LB medium, and finally the third stage is cells that achieve typical saturation in LB medium. We predicted that the number of mutants would increase from stage to stage as it is in the later stage that we detect suppressors at high frequency (Fig 13). In all cases the mutants per CFUs found in the \(\Delta\text{dinBdnaE915/pdinB(D103N)}\) strain isolated from the stages identified above was the same as \(\Delta\text{dinBdnaE915/pVector}\) (Table 1). From these data we can infer that if there is a hypermutator state, this is not detected by the assay performed.

**Table 1**

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<th>Rif(^R) mutants</th>
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<tr>
<td>pVector</td>
<td>1 ± 0.9</td>
<td>2x10(^9) ± 4x10(^8)</td>
</tr>
<tr>
<td>pdinB(D103N)</td>
<td>NF</td>
<td>1x10(^8) ± 4x10(^7)</td>
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<tr>
<td>1(^{st}) overnight(^b)</td>
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<tr>
<td>pVector</td>
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<tr>
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<tr>
<td>2(^{nd}) overnight(^b)</td>
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<tr>
<td>pdinB(D103N)</td>
<td>27 ± 10</td>
<td>2x10(^9) ± 3x10(^8)</td>
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\(^a\)=average of 30 colonies each
\(^b\)=average 3 isolate o/n with 10 replicates each
NF= none found
Discussion

Alternative activities of DinB include: roles in recombination, transcription, and a DNA replication "checkpoint" [17, 28, 44]. This checkpoint was originally identified in vivo as a loss of CFUs in the absence of DNA damage at high intracellular DinB concentrations (~15-72 times that of full SOS gene network induction) [17]. In vitro assays determined that DinB directly interacts with the β-processivity clamp, the α-catalytic subunit of DNA Pol III, or the DnaB helicase to slow or halt DNA replication [17, 22]. The loss of CFUs in vivo has also been suggested to be due to DNA double strand breaks resulting from DinB’s ability to incorporate 8-oxo-dG into undamaged DNA and their subsequent excision by DNA repair systems [20]. It has been argued that the high DinB concentrations needed to slow or halt DNA synthesis in previous studies [17, 22] could occur during SOS induction upon DNA damage. However, it is not yet known how a DinB replication brake would function in vivo in the presence of DNA lesions that may target DNA polymerases to the replicating fork and bring about DNA polymerase switching.

The experiments in this study were undertaken to examine the underlying mechanisms governing interactions between different bacterial DNA polymerases that occur during DNA polymerase exchange and/or upon activation of DNA replication checkpoints by DNA damage. For this purpose we chose DNA Pol IV (DinB), known to effect a checkpoint at high intracellular concentrations [17]. In addition, we also used dnaE915, [26, 27] an antimutator allele where there is evidence suggesting its activity is due to exclusion of TLS DNA polymerases, particularly DinB from the replication fork [28]. We hypothesized that DinB likely interacts differently with DNA Pol IIIα(915) than
DNA Pol IIIα, which would result in a detectable change in cellular sensitivity to DinB overexpression.

Indeed, we find the ΔdinBdnaE915 strain is more sensitive to DinB overproduction than ΔdinBdnaE+ (Fig 1). We find no difference between ΔdinBdnaE915 cells overproducing DinB+ or DinB(F13V), a variant incapable of incorporating 8-oxo-dG into DNA in vitro [20]. Our data indicates that 8-oxo-dG incorporation does not appear to play an important role in the loss of CFUs upon overproduction of DinB variants in the dnaE915 strain (Fig 1). This finding suggests that misincorporation of damaged bases during DNA synthesis likely contributes to DinB toxicity, but it is not the only reason to explain the phenotype.

We then asked the question of whether we could detect DinB dependent growth arrest in dnaE915 cells upon DNA damage treatment. Although a UV damage checkpoint occurs in cells with umuDC expressed from a plasmid [16, 45, 46], a DinB dependent growth arrest induced by DNA lesions has not been studied. We show that DinB has a preference for a DNA primer/template junction containing a 3-meA lesion analogue compared to undamaged DNA in vitro (Fig 3). Therefore, lesions might help target DinB to the DNA in vivo, and elevate its local concentration above a threshold necessary for a checkpoint. dnaE915 cells carrying either chromosomal dinB(D103N) or plasmid borne native dinB under its own promoter are hypersensitive to MMS treatment, an agent that forms 3-meA lesions (Fig 2). The dinB(D103N)dnaE915 strain is uniquely hypersensitive to reagents generating DinB cognate lesions (MMS or NFZ) and not to UV irradiation, indicating that lesion specificity plays an important role in targeting DinB(D103N) to the stalled replication fork. Because DinB(D103N) is catalytically inactive, the growth arrest
likely results from direct or indirect protein-protein interactions between DNA Pol IIIα and DinB(D103N) in the presence of DNA damage and not catalytic functions (e.g. incomplete TLS [37] or incorporation of 8-oxo-dG [20]).

Notably, we find an antagonistic genetic interaction between dnaE915 and dinB(D103N) expressed from its own promoter on a low copy number plasmid. The DNA damage independent growth defects are manifested as aberrant colony morphology and inability to reach saturation in liquid medium (Fig 5). We infer that the observed synthetic sickness is due to replication stress because the SOS gene network is induced in these cells in the absence of exogenous sources of DNA damage (Fig 6).

We find several intragenic dinB mutations (Fig 13) that rescue dnaE915 (or dnaE+) strains from DinB-dependent growth arrest (Figs 10, 11, 14, 15, and 16), indicating that these residues are responsible for protein-protein interactions that lead to cell growth arrest or are important for DinB protein folding and stability. However, although these mutations inhibit growth defects, we have found two variants that are still able to carry out TLS in vivo (F292Y or V7G) or in vitro (F292Y) (Figs 15). Curiously, the in vivo activity of these two variants is contingent on both the dnaE allele and the type of DNA damage (Fig 15). This finding suggests that DinB+ and its variants likely interact differently with either DNA Pol IIIα or DNA Pol IIIα(915). The interactions between DNA polymerases are further nuanced by the lesions the DNA polymerases encounter (Fig 15).

From the evidence presented in this report we propose a model to explain how DinB effects an in vivo growth arrest in the presence of DNA damage (Fig 18). It appears that lesions play at least two roles by both inducing the transcription of dinB and its
alleles [25] and by targeting DinB to the fork (Fig 3). DinB then accesses the replication fork, likely through interactions with the β-clamp [39] or other replication proteins, and could carry out TLS, allowing resumption of DNA replication and thus cell survival. Alternatively, if the DinB variant cannot bypass the lesion or if it otherwise prevents DNA Pol IIIα from resuming synthesis (e.g. a high local DinB concentration that excludes Pol IIIα from β-clamp or helicase), the replication fork would remain stalled and growth arrest would occur (Fig 18).

![Diagram](image)

**Figure 18. A model of DinB-mediated growth arrest resulting from DNA damage treatment.** DNA Pol III α-subunit is represented by the white shape. β-clamp is represented as a donut shape encircling the DNA. DinB(D103N) is depicted as a black shape. The DinB(D103N) shapes are clumped together to illustrate increase in local concentration at the replication fork and is not meant to suggest they bind to one another. Lesions are depicted as an “X” on the DNA. See text for details.
Taking these data together, the *dnaE915* genetic system that we have utilized allows for the identification of an *E. coli* DinB dependent growth arrest upon DNA damage treatment. We also identified *dinB* intragenic mutations that suppress the various DinB dependent growth arrest phenotypes and discovered they are mostly localized in a specific area of DinB. We infer this area of DinB likely interacts directly or indirectly with DNA Pol IIIα and/or other replication proteins and is important for the interplay between DNA polymerases in the cell. Further *in vitro* investigations utilizing the DinB variants we have identified will aid in determining what areas of the protein are important for specific protein-protein interactions that lead to polymerase switching or cytotoxicity.
References


Chapter 3

DNA Pol IV plays a novel TLS independent role in cell survival upon DNA damage treatment in *Escherichia coli*.

Abstract

All cells use multi-protein DNA polymerase complexes to synthesize a high fidelity copy of a cell’s genome. However, when they encounter lesions on the template DNA they are unable to continue with DNA synthesis and replication stalling occurs. To avoid lethality associated with stalled replication forks, specialized translesion (TLS) DNA polymerases insert deoxynucleotides opposite to and extend from replication-stalling DNA lesions on the template DNA. *Escherichia coli* DNA Pol IV (DinB) is of particular interest because of its evolutionary conservation and its high basal intracellular concentration. The relatively high concentration of DinB in the cell when compared to other polymerases could be due to its role in the tolerance of low levels of endogenous DNA damage generated by normal metabolism. We hypothesized that ΔdinB strains that are highly sensitive to DNA damage because of mutations in *recA*, *lexA*, or other repair pathways would allow us to detect whether DinB bypass deficient variants could rescue these cells from lethality upon DNA damage treatment. To separate the importance of DinB’s replication of undamaged template from its replication of lesion containing templates, we utilized DinB(Y79A) or DinB(F13V) variants that are unable to bypass alkylating or $N^2$-dG lesions, but retain catalytic activity on undamaged DNA. We find expression of *dinB(Y79A)* and *dinB(F13V)* but not *dinB(D103N)*, encoding a catalytically inactive DinB variant, rescues these highly sensitive cells from treatment with DNA damaging agents. These data suggest that catalytic activity, but not TLS activity, is required for
cellular resistance to treatment. Furthermore, ΔrecA cells carrying pdinB(Y79A), an allele shown to be a mutator upon nitrofurazone treatment in recA+ cells, have a low mutant frequency upon treatment with nitrofurazone or methyl methanesulfonate. These results indicate DinB has a novel role that is due to the largely error free synthesis of undamaged DNA and independent of SOS induction, recA homologous recombination, or base excision repair. Our results support the previously reported notion that DNA lesions target DinB to the stalled replication fork.

Introduction

DinB (DNA Pol IV) is a highly conserved translesion synthesis (TLS) DNA polymerase found in all domains of life [1]. Its ubiquity and the fact it is the second most abundant DNA polymerase in E. coli cells after the gap filling DNA Pol I (250 nM vs 400 nM) [1, 2] suggest it has one or more roles vital to the cell. Indeed, the other two TLS polymerases of E. coli (Pol II; 40 nM and Pol V; 15 nM) and the replicative polymerase (Pol III; 15 nM) are found at significantly lower basal intracellular levels [2]. Much has been discovered regarding the role of DinB in lesion bypass and mutagenesis [1, 3-8]. Cells lacking dinB (ΔdinB) are sensitive to nitrofurazone (NFZ) [9] and alkylating agents such as methyl methanesulfonate (MMS) [10]. These compounds generate diverse lesions (NFZ; $N^2$-dG or MMS; 3-meA) that are known to stall the replicative DNA polymerase Pol III but are bypassed both in vivo and in vitro by DinB [3, 9]. The introduction of a low copy number plasmid expressing dinB+ under its native promoter is able to complement the NFZ or MMS sensitivity of ΔdinB strains [5, 9, 10]. Changes in specific catalytic residues (F13 or Y79) render DinB TLS deficient [9, 11], i.e. able to synthesize undamaged DNA but unable to both insert a nucleotide opposite a
lesion and extend from it. The expression of either TLS deficient DinB(F13V) or DinB(Y79A) variants in ∆dinB cells does not rescue these cells from NFZ or MMS treatment [5].

Alternative activities of DinB other than its ability to insert a nucleotide opposite specific DNA lesions include: roles in recombination, transcription, and a DNA replication "checkpoint" [12-14]. All three DNA TLS polymerases (Pol II, Pol V, and DinB) are also required for fitness during long term stationary phase of growth, even in the absence of exogenous DNA damage [15]. Their requirement has been hypothesized to be dependent on the bypass of lesions generated by cellular metabolism or to the generation of potentially beneficial mutations resulting from the low fidelity of TLS polymerases [16]. DinB’s importance in the tolerance of endogenous DNA damage could also explain its high basal level [1, 2, 8], allowing cells to manage lesions quickly upon resumption of DNA replication as cells exit non-growing conditions. However, it is not known if DinB TLS activity is specifically required for survival in a non-growing condition, such as stationary phase. We undertook this investigation to determine whether replication of undamaged DNA by DinB rescues survival during DNA damage treatment, permitting us to learn how cells respond to DNA damage processes that are still poorly understood. It will also give further insight into conditions and proteins required for DinB to access the replication fork through the still unclear process of polymerase switching, a RecA-dependent process in vitro [17]. A variety of strains sensitive to DNA damage [1] that are deficient in both recombination and the induction of the SOS DNA damage response, and/or base excision repair (BER) allowed detection of TLS deficient DinB dependent changes in cell survival upon treatment with DNA damaging agents.
We show that expression of \textit{dinB(Y79A)} or \textit{dinB(F13V)}, but not the catalytically inactive \textit{dinB(D103N)}, rescues these highly sensitive cells from treatment with DNA damaging agents, suggesting that catalytic activity, but not TLS, is required for cellular resistance to treatment. Furthermore \textit{\Delta recA} cells with \textit{pdinB(Y79A)}, an allele shown to be a mutator upon NFZ treatment in \textit{recA} \textsuperscript{+} cells [5], have low mutant frequency upon treatment with NFZ or MMS. These results indicate DinB plays a part in the cellular response to DNA damage that is likely due to the largely error free synthesis of undamaged DNA and independent of \textit{recA} mediated recombination, induction of the SOS damage response, or BER.

\textbf{Materials and Methods}

\textbf{Bacterial Strains and Plasmids.} Bacterial strains and plasmids are listed in Appendix C. Plasmids were introduced into CaCl\textsubscript{2} chemically competent cells by transformation [18]. Chromosomal deletions or gene alleles were moved between different \textit{E. coli} strains via P1 transduction [18].

\textbf{Survival Assays.} Cultures were grown to saturation in liquid LB. Ampicillin (Amp, 200 \textmu g/mL; Sigma) was added to the LB if plasmid maintenance is required. Serial dilutions of saturated cultures were treated with varying concentrations of methyl methanesulfonate (MMS; 0.04 and 1 mM; Acros Organics), nitrofurazone (NFZ; 0.3, 1.8, or 5 \textmu M; Sigma), or were irradiated at a UV (254 nm) intensity of 1.2 J/m\textsuperscript{2}.

\textbf{DNA Polymerase extension assays.} Native DinB variants were purified and assayed as before [3] with 17.5 \textmu M of DinB(F13V) or DinB(Y79A) per reaction. Template and primer oligonucleotides used are in Appendix C.
Mutation Assays. We carried out the same procedure as before (Chapter 1; [5, 19]) to identify DNA damage (NFZ; 0.3 μM or MMS; 0.04 mM) induced mutants. Experiments with three independent isolates were carried out until at least 362 colonies were screened per isolate.

Results and Discussion

We constructed strains with chromosomal copies of TLS deficient dinB alleles (dinB(F13V) or dinB(Y79A)) [5, 9, 11] and a deletion of recA (ΔrecA) that would render cells deficient for both homologous recombination and induction of the SOS gene network [1], thus sensitizing them to DNA damage. In vivo TLS activity of DinB is measured as the ability to rescue the survival of ΔdinB cells upon treatments that result in DinB cognate lesions (i.e. NFZ or MMS) [5, 9]. Neither TLS deficient DinB variant rescues recA+ΔdinB cells upon treatment with concentrations of NFZ or MMS that result in a tenfold killing of the isogenic ΔdinB strain [5]. Therefore, any survival observed in ΔrecA cells carrying these TLS deficient DinB variants when compared to an isogenic strain with a deletion of dinB (ΔdinB) is likely independent of DinB’s TLS activity. We find that when compared to ΔrecAΔdinB, ΔrecA cells with a chromosomal copy of dinB(F13V) (ΔrecAdinB(F13V)) are more resistant to NFZ treatment while ΔrecA cells carrying a chromosomal copy of dinB(Y79A) (ΔrecAdinB(Y79A)) are more resistant to NFZ or MMS treatment (Fig 1A). ΔrecAΔdinB strains with dinB(F13V) or dinB(Y79A) expressed from their native promoter on a low copy number plasmid display the same survival as when either allele is expressed from the chromosome (Fig 1B). Remarkably, the recA+ counterparts, i.e. recA+ΔdinB cells with dinB(F13V) expressed from the identical low copy number plasmid used in these studies are hypersensitive to NFZ [5, 9],
and the isogenic strain expressing \textit{dinB(Y79A)} is hypersensitive to both NFZ and MMS [5]. These data suggest that cellular hypersensitivity to NFZ or MMS [5] is likely dependent on RecA, since both TLS deficient variants can rescue Δ\textit{recAΔdinB} cells upon DNA damage.

DinB(F13V) and a variant similar to DinB(Y79A) (DinB(Y79L)) are unable to bypass \textit{N}^2-dG lesions \textit{in vitro} [9, 11]. It is unknown if either variant could bypass a 3-meA analogue (3-deaza-3-methyl-adenine; 3-dmeA) \textit{in vitro}. We find that \textit{in vivo}, only DinB(Y79A) rescues cells from MMS lethality (Fig 1). Perhaps this is due to this variant’s ability to bypass 3-meA-lesion \textit{in vitro}. However we find that DinB(Y79A)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{	extbf{TLS deficient DinB variants rescue Δ\textit{recA} cells upon DNA damage treatment in a lesion specific manner.} (A) Chromosomal borne DinB(F13V) and DinB(Y79A) rescue Δ\textit{recA} cells treated with NFZ (0.3 µM; white bars), but only DinB(Y79A) rescues these cells upon MMS treatment (0.04 mM; gray bars). No effect in the survival of Δ\textit{recA} cells is observed upon treatment with UV (1 J/m$^2$; black bars). (B) As in (A) but with plasmid borne DinB variants. The catalytically inactive DinB(D103N) does not rescue Δ\textit{recAΔdinB} cells in NFZ or MMS treatment. Error bars represent the standard deviation of the mean from at least 3 independent isolates.}
\end{figure}
does not bypass the 3-meA analogue \textit{in vitro} (Fig 2), and consistent with \textit{in vivo} data, DinB(F13V) is also unable to do so.

![Figure 2. DinB(Y79A) or F13V cannot bypass 3-meA analogue \textit{in vitro.}](image)

(A) Schematic of a primer template junction containing an adenine or 3-deaza-3-methyl-adenine (3-dMeA). The HEX fluorescent tag is depicted as a star. (B) Standing start DNA extension assays with 25 nM of undamaged or lesion containing templates (same as those shown in Fig 2A). DinB(F13V) and DinB(Y79A) can only synthesize DNA on undamaged template and not 3-deaza-3-methyl-adenine (3-dMeA).

The observed rescue shown in Fig 1 was independent of TLS, however, it is unclear if DinB’s ability to replicate undamaged DNA is required or if the rescue is perhaps the result of protein-protein interactions. We introduced a low copy number plasmid encoding the catalytically inactive DinB variant (DinB(D103N)) from its native promoter into $\Delta recA \Delta dinB$ cells and assayed for NFZ or MMS resistance. We find these cells survive either treatment as an isogenic strain carrying the vector (Fig 1B), indicating the ability to replicate an undamaged template is likely required. The importance of the catalytic activity of DinB suggested it indeed was replicating DNA. We then sought to determine if the \textit{in vivo} DNA synthesis by DinB(Y79A) would generate mutations during NFZ treatment as it does in isogenic $recA^+$ cells [5]. We chose the
ΔrecAΔdinB/pdinB(Y79A) strain to test this hypothesis, predicting it would have the highest mutator activity. However, out of over 1000 colonies screened for each treatment, we find only one NFZ (0.3 µM) induced mutant (0.01% ± 0.005% SEM) and no MMS (0.04 mM) induced mutants. This is lower than the mutant frequency (1% ± ≤0.05% SEM in NFZ or 0.2% ± 0.008% SEM in MMS) found in recA+ strains treated with levels of DNA damaging agents that result in comparable levels of killing [5] to those used here. This indicates that DinB(Y79A) rescues cells without generating DNA damage induced mutations at these levels of DNA damage treatment.

Both NFZ and MMS generate lesions that are bypassed in vivo by DinB [3, 9, 10], however, because its TLS activity is dispensable for rescue under the current conditions of DNA damage treatment, it is plausible DinB may rescue cells treated with agents that do not form cognate lesions, such as UV irradiation [1]. We find DinB(F13V) and DinB(Y79A) have no effect on survival compared to ΔdinB upon UV treatment, whether they are expressed from the chromosome or plasmid (Fig 1). Therefore it is likely DinB is able to access undamaged DNA after it is targeted to the stalled replication fork specifically by its cognate lesions. There are several possibilities as to how this lesion specific rescue occurs. It is possible that in the absence of recA, these variants may have some in vivo TLS activity that is dependent on interactions with various replicative proteins. Alternatively, these variants may help localize other TLS DNA polymerases or DNA repair proteins to the stalled replication fork. Examples of these are DNA Pol II or Pol V and base excision repair (BER) proteins involved in the repair of lesions in the template DNA. Finally, replication of undamaged DNA by DinB as part of recA independent recombination pathways could also increase cell survival.
To test whether the TLS deficient DinB variant mediated survival was due to the absence of recA, we constructed a ∆dinB strain that was recA⁺ but deficient in SOS induction due to an uncleavable variant of the lexA suppressor (lexA₃) and thus sensitive to DNA damage [1]. RecA physically interacts with DinB and controls its catalytic activity, raising the possibility it somehow also inhibits the synthesis of undamaged DNA by TLS deficient variants upon DNA damage treatment. However, we find even in the presence of recA, the plasmid borne dinB(Y79A) rescues lexA₃∆dinB cells from MMS treatment (Fig 3) when compared to the isogenic strain carrying the vector. However, these alleles fail to rescue lexA₃∆dinB cells upon NFZ treatment (Fig 3). Future studies must determined if this is due to recA modulating the DNA replication by TLS deficient
DinB variants in a lesion specific manner or if the concentration of NFZ is above that at which either variant can offer any rescue (0.3 µM for ΔrecAΔdinB vs 1.8 µM for lexA3ΔdinB).

The lexA3ΔdinB strain is sensitive to DNA damage compared to wild-type cells, however, it is more resistant than the ΔrecA strain used above and requires higher concentrations of NFZ or MMS to observe the same level of killing. There may be a “tipping point” between 0.3 µM and 1.8 µM NFZ where the amount of DNA lesions or other effects of treatment render these TLS deficient variants unable to rescue cells.

Nucleotide excision repair (NER) is a major part of the cellular response to NFZ treatment [20]. Perhaps a lexA3 strain that is NER deficient, such as lexA3ΔuvrAΔdinB, would be sensitive to a dosage of NFZ that is low enough to allow for dinB(F13V) or dinB(Y79A) expression to rescue upon treatment. Although the data in Fig 3 does not clarify the role of recA in cell rescue upon NFZ treatment by TLS deficient DinB variants, the dinB(Y79A) dependent rescue of MMS treatment is independent of recA. This indicates the presence of RecA alone does not interfere with processes that allow for DinB dependent survival rescue of cells treated with DNA damaging agents.

It was possible that other TLS polymerases are required for DinB to rescue cells. In yeast, multiple TLS polymerases are involved in the in vivo bypass of 3-meA lesions [21]. In E. coli, resistance to hydroxyurea (HU) that is dependent on a truncated allele (umuC122) of the umuC gene that encodes the catalytic subunit of Pol V also requires DinB [22], indicating these two TLS polymerases interact at times to increase cell survival during replication stress. UmuC and a truncated dimer of UmuD (UmuD’) make up the Pol V multiprotein complex (UmuD’2UmuC) [23]. Additionally, both forms of
UmuD are known to interact with various replicative proteins and regulate DinB’s mutagenic potential [4]. However, because the RecA nucleoprotein filament is required for the autocleavage of UmuD to a truncated UmuD’ [24] the amount of catalytically active Pol V in ΔrecA cells is likely low or nonexistent. A ΔrecAΔdinB strain with a deletion of the umuDC operon that encodes the components of Pol V (ΔrecAΔumuDCΔdinB) was constructed to determine if UmuD, UmuC, or DNA Pol V is required for DinB dependent resistance to DNA damage treatment. Preliminary data reveals ΔrecAΔumuDCΔdinB cells carrying plasmid borne dinB(F13V) or dinB(Y79A) are resistant to NFZ or MMS treatment when compared to cells carrying the vector (data

![Graph](image.png)

**Figure 4.** DinB(Y79A) dependent rescue during treatment with MMS is independent of alkA or tag base excision repair genes. Plasmid borne DinB(Y79A) rescues recAΔalkAΔtagΔdinB cells during MMS treatment (0.04 mM; grey bars) but fails to rescue the same strain upon NFZ treatment at the concentration required to observe 10-fold killing (5 µM; white bars) of cells with the vector. Error bars represent the standard deviation of the mean from at least 3 independent isolates.
not shown). Thus, the observed rescue of cells by these DinB variants is independent of the UmuD or UmuC components of Pol V. However, future studies will have to be conducted with a deletion of the gene encoding Pol II (ΔpolB) before DinB recruitment of other TLS polymerases can be ruled out.

We next tested the activity of dinB(F13V) and dinB(Y79A) in a strain that is SOS proficient (recA+ and lexA+) but deficient for the BER genes involved in the repair of alkylating lesion generated by MMS [10]. We hypothesized that if DinB is targeting BER to lesions and in this way rescuing cells from DNA damaging agents, the previously observed survival (Figs 1 and 3) would disappear in the BER deficient strain. Additionally this strain would indicate if upregulation of any SOS gene, including the dinB alleles, abolishes the rescue observed in ΔrecA or lexA3 SOS deficient strains (Figs 1 and 3). The plasmid encoding dinB(Y79A) was introduced by transformation into recA+ strains with deletions of alkA, tag, and dinB genes (recA+ΔalkAΔtagΔdinB). Indeed, these cells are resistant to MMS when compared to cells carrying the vector (Fig 4). However, like the lexA3 strain, recA+ΔalkAΔtagΔdinB cells expressing dinB(Y79A) do not survive NFZ treatment when compared to cells carrying the vector. This may be due to the inhibition of DinB activity by RecA. Preliminary evidence suggests an isogenic strain lacking recA (ΔrecAΔalkAΔtagΔdinB) and expressing plasmid borne dinB(F13V) or dinB(Y79A) has similar survival trends to those observed in the ΔrecAΔdinB background (Fig 1) and thus survive better than an isogenic strain with the vector (data not shown). These data indicate alkA and tag are not required for DinB(Y79A) dependent NFZ or MMS resistance and DinB is likely not targeting these proteins to the stalled replication
fork to eliminate DNA lesions. These data also suggest that RecA does not inhibit TLS deficient DinB variants from effecting survival upon DNA damage treatment.

There is evidence that a variant of the catalytic $\alpha$-subunit of the replicative Pol III (Pol III$\alpha$(915)), coded by dnaE915, interacts differently with DinB that the wild type DNA Pol III$\alpha$ (Chapter 2; [13]). It has been hypothesized that the antinmutator properties of the dnaE915 allele is due to the exclusion of DinB by DNA Pol III$\alpha$(915) from participating in DNA synthesis during double strand break repair [13]. We reasoned if DinB(F13V) or DinB(Y79A) are replicating undamaged DNA as part of a homologous recombination pathway that is responding to DNA damage, the rescue would be

Figure 5. The dnaE915 allele does not prevent TLS deficient DinB variants from rescuing in DNA damage treatment. (A) Both plasmid borne DinB(F13V) and DinB(Y79A) rescue $\Delta$recA$\Delta$dinBdnaE915 cells treated with NFZ (0.3 $\mu$M; white bars). Rescue in MMS treatment is observed in cells with DinB(Y79A) (0.04 mM; gray bars). The dinB alleles have no effect in the survival of cells treated with UV (1 J/m$^2$; black bars). (B) The dnaE915 allele has no effect on plasmid borne DinB(Y79A) dependent rescue of MMS treated lexA3$\Delta$dinB cells. Error bars represent the standard deviation of the mean from at least 3 independent isolates.
abolished in dnaE915 cells. However, we find ∆dinBdnaE915 cells with either ∆recA or lexA3 alleles are rescued by plasmid borne dinB(F13V) or dinB(Y79A) (Fig 5) to the same degree as an isogenic dnaE+ strain (compare Figs 1 and 3 to 5). This would suggest the observed resistance is not part of homologous recombination mediated double strand break repair, however, the DinB variants may be acting in an alternate recombination pathway to effect cellular survival. Alternatively, as in Chapter 2, these results could indicate that, although the dnaE915 allele does lessen DinB mutagenesis [13], it does not abolish the ability of DinB variants to access the replication fork and effect either DNA damage dependent cellular sensitivities or resistances.

In summary we have identified a TLS independent role for DinB in cellular survival upon treatment with DNA damaging agents. Although TLS is dispensable for this rescue, the ability to replicate undamaged DNA is required (Fig 1). Furthermore this rescue is only present during treatment with agents that generate DinB cognate lesions (MMS and NFZ but not UV) (Fig 1). This suggests lesions target DinB to the replication fork where the TLS deficient variants have access to undamaged DNA. This function is also independent of BER (Fig 4) and the subunits of Pol V (UmuD or UmuC), demonstrating DinB is unlikely to localize BER mechanisms or Pol V to rescue cells. It is still uncertain if DinB may localize other excision mechanisms such as nucleotide excision repair (NER), a major component of the cellular response to NFZ treatment [20] or the Pol II TLS polymerase. Although the effect of the TLS deficient DinB variants on survival does not require recA dependent recombination, it is unknown if other repair pathways such as the sbcCD pathway are required [1, 25]. Another intriguing possibility
is that DinB may be taking part in replication restart and could be dependent upon \textit{priA} [26].

The role for DinB that we have uncovered in this study could explain its conservation throughout all domains of life and its high intracellular concentration in \textit{E. coli} cells. \textit{E. coli} strains missing one or more of their TLS polymerases are at a competitive disadvantage in long term stationary phase when grown together with wild type cells, even in the absence of exogenous DNA damage [15]. Indeed TLS polymerases other than DinB may also replicate undamaged DNA after being recruited to the fork in a lesion dependent manner. It would be interesting to investigate whether cells carrying TLS deficient \textit{dinB} alleles are as competitively disadvantaged as strains with a \textit{dinB} deletion in long term stationary competition experiments. Perhaps this enigmatic requirement for DinB in long term stationary phase [15] is due to the replication of undamaged DNA in the presence of endogenous DNA damage.
References

Conclusion

In this work we have identified the role played by DinB’s active site residues in lesion bypass fidelity, a function previously unknown. We studied a highly conserved aromatic residue triad F12, F13, and Y79 in the DinB active site. By changing these conserved residues to ones of different size and polarity we determined that the different DinB variants affected cellular survival during DNA damage treatment as well as the frequency of DNA damage induced mutants.

All three residues are important for in vivo DinB bypass of both $N^2$-dG and alkylating lesions. As previously reported [1, 2] we find expression of the TLS deficient DinB(F13V) or DinB(Y79A) variants in $\Delta$dinB cells from their native promoter on a low copy number plasmid causes hypersensitivity to NFZ treatment (i.e. cells survive worse than cells carrying the vector). However, we discovered cells carrying pdinB(Y79A) but not pdinB(F13V) are hypersensitive to MMS (Chapter 1, Fig 5), suggesting that the DinB active site adjusts to lesions and although these residues are important for TLS, they may play lesion specific roles. When compared to the in silico model of DinB, the Pol $\kappa$ crystal structure shows that the aromatic triad is identical in conformation (DinB F12, F13 and Y79 are homologous to Pol $\kappa$ F111, Y112, and Y174; Chapter 1, Fig 1). Thus, this triad of aromatic residues in the DinB active site might be playing similar roles in DinB homologues especially regarding fidelity.

Interestingly we find that $\Delta$dinB cells carrying a plasmid encoding a catalytically inactive DinB variant (D103N) are hypersensitive to both NFZ and MMS treatments (Chapter 1, Fig 3). We hypothesized DinB(D103N) hypersensitivity could be due to
interactions with the replicative Pol III and that the growth arrest is the result of a DNA damage inducible replication checkpoint.

We next examined the underlying mechanisms governing interactions between different bacterial DNA polymerases that occur during DNA polymerase exchange and/or upon activation of DNA replication checkpoints by DNA damage. This checkpoint was originally identified in vivo as a loss of CFUs in the absence of DNA damage at high intracellular DinB concentrations (~15–72 times that of full SOS gene network induction) [3]. However, it was not known how a DinB replication brake would function in vivo in the presence of DNA lesions that may target DNA polymerases to the replicating fork and bring about DNA polymerase switching. For this purpose we continued our structure/function analysis of DinB, in conjunction with dnaE915, [4, 5] an antimutator allele encoding Pol IIIα(915). Because this variant likely interacts differently with DinB than DNA Pol IIIα [6], we hypothesized there would be a detectable change in cellular sensitivity to DinB overexpression in the dnaE915 strain.

Indeed, the ΔdinBdnaE915 strain is more sensitive to DinB overproduction than ΔdinBdnaE+ (Chapter 2, Fig 1). We also detected DinB dependent growth arrest upon DNA damage treatment in dnaE915 cells bearing a chromosomal copy of dinB(D103N) or dinB+ expressed from its own promoter in a low copy number plasmid (Chapter 2, Fig 2). The dinB(D103N)dnaE915 strain is uniquely hypersensitive to reagents generating DinB cognate lesions (MMS or NFZ) and not to UV irradiation, indicating that lesion specificity plays an important role in targeting DinB(D103N) to the stalled replication fork. Because DinB(D103N) is catalytically inactive, the growth arrest likely results from direct or indirect protein-protein interactions between DNA Pol IIIα and DinB(D103N)
in the presence of DNA damage and not catalytic functions (e.g. incomplete TLS [1] or incorporation of damaged nucleotides [7]). Indeed, even in the absence of DNA damage treatment, we find an antagonistic genetic interaction between dnaE915 and pdinB(D103N) that is manifested as aberrant colony morphology and inability to reach saturation in liquid medium (Chapter 2, Fig 5). We infer that the observed synthetic sickness is due to replication stress because the SOS gene network is induced in these cells in the absence of exogenous sources of DNA damage (Chapter 2, Fig 7).

Due to the severe selective pressure to generate suppressor mutations in the above genetic situations, we found several intragenic dinB mutations that rescue dnaE915 or dnaE+ strains from DinB-dependent growth arrest (Chapter 2, Figs 10, 11, 14, 15, and 16). However, although these mutations inhibit growth defects, we have found two variants that are still able to carry out TLS (Chapter 2, Fig 15). Curiously, the in vivo activity of these two variants is contingent on both the dnaE allele and the type of DNA damage (Chapter 2, Fig 15). This finding suggests that DinB+ and its variants likely interact differently with either DNA Pol IIIα or DNA Pol IIIα(915). The interactions between DNA polymerases are further nuanced by the lesions the DNA polymerases encounter (Chapter 2, Fig 15). Future in vitro investigations employing the DinB variants we have identified in conjunction with other replicative proteins (i.e. Pol IIIα, β-clamp, or DnaB helicase) will aid in determining what areas of the protein are important for polymerase switching or for growth arrest.

From the evidence presented in Chapters 1 and 2, we propose a model to explain how DinB effects an in vivo growth arrest in the presence of DNA damage (Chapter 2,
Fig 18). It appears that lesions play at least two roles by both inducing the signaling cascade that results in elevated transcription of DNA damage response genes including \textit{dinB} and its alleles \cite{8} and by targeting DinB to the stalled replication fork (Chapter 2, Fig 3). DinB then accesses the replication fork, likely through interactions with the β-clamp \cite{9} or other replication proteins, and could carry out TLS, allowing resumption of DNA replication and thus cell survival. Alternatively, if the DinB variant cannot bypass the lesion or if it otherwise prevents DNA Pol IIIα from resuming synthesis (e.g. a high local DinB concentration that excludes Pol IIIα from β-clamp or helicase), the replication fork would remain stalled and growth arrest would occur.

The investigation in Chapter 3 revealed a TLS independent role for DinB in cellular survival during DNA damage treatment. To detect DinB dependent changes in cellular tolerance of DNA damage, we used strains that were deficient for \textit{recA} dependent recombination, SOS gene network induction, or base excision repair. TLS deficient DinB variants (DinB(F13V) or DinB(Y79A)) but not the catalytically inactive DinB(D103N) expressed from the chromosome or low copy number plasmids rescue Δ\textit{recA}Δ\textit{dinB} cells during MMS and/or NFZ treatment (Chapter 3, Fig 1), revealing DinB’s ability to replicate undamaged DNA since its TLS activity is not required. The TLS deficient DinB variants also rescue Δ\textit{recA}Δ\textit{dinB}Δ\textit{umuDC} cells, eliminating the possibility that DinB works with Pol V to bypass the lesions or that the components (UmuD or UmuC) alone are required for rescue. However, future studies will have to address the possibility that Pol II plays a part in cell survival. Surprisingly, although TLS activity is dispensable for rescue, we only see this feature in the presence of agents that generate DinB cognate
lesions (MMS and NFZ but not UV) (Chapter 3, Fig 1). This suggests lesions target DinB to the replication fork where the TLS deficient variants then access undamaged DNA.

Cells that are hypersensitive to MMS due to deficiencies in base excision repair but proficient in SOS induction and recombination (recAΔalkAΔtagΔdinB) are also rescued by pdinB(Y79A) (Chapter 3, Fig 4), demonstrating this DinB variant does not rescue cells by localizing repair mechanisms to the stalled fork. Furthermore, it also suggests that this phenotype can be observed in recA+ strains.

Taken together, these structure/function studies have shed light onto the importance of various residues and areas of DinB in its varied roles in the cell. We have shown here that the high fidelity of DinB is apparent upon alkylation damage, an inescapable and pervasive form of DNA damage, even with DinB variants that perform in vivo error-prone NFZ-induced lesion bypass. We have identified an E. coli DinB dependent growth arrest upon DNA damage treatment and various intragenic mutations that suppress this growth arrest. In addition, we propose that it is the nature of the lesion that localizes DinB to the replication fork leading to DNA polymerase exchange with the replicative DNA polymerase when it has stalled. A novel TLS independent activity of DinB upon DNA damage treatment has also been described here. This information is vital in gaining further insights into the varied and sometimes cryptic mechanisms of DNA polymerases, an important component in medically relevant processes such as antimicrobial resistance [10, 11] and cancer [12-16].
Reference

### Appendix A - Chapter 1 strain, plasmid, and oligonucleotide list

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<th>Strains</th>
<th>Description</th>
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### Plasmids

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### Oligonucleotides

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<td>F13VAβ</td>
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**References**

### Appendix B - Chapter 2 strain, plasmid, and oligonucleotide list

<table>
<thead>
<tr>
<th>Description</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>P90C</td>
<td>F- ara Δ(lac-pro)XII thi [1]</td>
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<td>ΔdinB</td>
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<tr>
<td>RWB1581</td>
<td>zae::Tn10dcam from SMR6114 [3] replacing dnaE+ This work</td>
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<tr>
<td>RWB2028</td>
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<td>RWB2370</td>
<td>As RWB2028 but with (ΔTLS) ΔpolB::Kan and ΔumuDC::cat This work</td>
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<td>RWB2436</td>
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<td>RWB2367</td>
<td>As RWB1581 but with dinB(F13V) replacing dinB+ This work</td>
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<td>RWB2373</td>
<td>As RWB1581 but with dinB(Y79A) replacing dinB+ This work</td>
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<td>RWB2839</td>
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<td>RWB2846</td>
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<td>ΔdinB::Kan was inserted into the E486 strain [5] carrying the temperature sensitive dnaE486 This work.</td>
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<td>BL21-Al ΔdinB</td>
<td>BL21-Al ΔdinB::Kan [7]</td>
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<td><strong>Plasmids</strong></td>
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<td>pWSK29</td>
<td>pSC101 replicon with pBluescript II SK+ multiple cloning site, AmpR [8]</td>
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<tr>
<td>pYG768</td>
<td>pWSK29 with dinB+ under its native promoter [9]</td>
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<tr>
<td>pYG768(D103N)</td>
<td>As pYG768 but with dinB(D103N) [10]</td>
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<td>pYG768(F13V)</td>
<td>As pYG768 but with dinB(F13V) [11]</td>
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<td>pYG768Δβ</td>
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<td>pYG768(D103N)Δβ</td>
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<td>pYG768(V288G)</td>
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<tr>
<td>pYG768(F292Y)</td>
<td>As pYG768 but with dinB(F292Y) This work</td>
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<td>pYG768(V7G)</td>
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<td>pBAD18(dinB+)</td>
<td>dinB+ with ara promoter inserted with KpnI and XbaI restriction sites into pBAD18 [12] This work</td>
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pBAD18(*dinB*(D103N)) As pBAD18(*dinB*) but with *dinB*(D103N) This work
pBAD18(*dinB*(F13V)) As pBAD18(*dinB*) but with *dinB*(F13V) This work
pBAD18(*dinB*(Y79A)) As pBAD18(*dinB*) but with *dinB*(Y79A) This work
pBAD18(*dinB*(F292Y)) As pBAD18(*dinB*) but with *dinB*(F292Y) This work
pBAD18(*dinB*(V7G)) As pBAD18(*dinB*) but with *dinB*(V7G) This work
pET11T(*dinB*(D103N)) As pET11T(*dinB*) but with *dinB*(D103N) [7]
pET11T(*dinB*(F292Y)) As pET11T(*dinB*) but with *dinB*(F292Y) This work

### Oligonucleotides

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<th>Reverse Primer</th>
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<td>GAACCGTTTGCACGTGAATGAGGCTTA</td>
<td>CAGTGACAACGTTCAATGCGCAG</td>
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<tr>
<td>F13V</td>
<td>GATATGGAACGTGCTTTGTCCCGCAGTGG</td>
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**Undamaged control template for primer**

**GCT CGT CAG ACG ATT TAG AGT CTG CAG TG** [7]
Lesion containing template for primer extension assay

GCTCGTCAGACG/3-deaza-3-methylA/TTTAGAGTCTGCAGTG [7]

Fluorescently labeled primer for standing start primer extension

/HEX/CACTGCAGACTCTCTAAA [7]

References

### Appendix C - Chapter 3 strain, plasmid, and oligonucleotide list

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
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<td>P90C</td>
<td>F- ara (\Delta(lac-pro)_{11}) thi</td>
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<tr>
<td>RWB1580</td>
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<td>RWB3173</td>
<td>As P90C but with (\Delta\text{recA}::\text{Kan})</td>
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<td>RWB2182</td>
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<td>pSC101 replicon with pBluescript II SK(^+) multiple cloning site, Amp(^R)</td>
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</tr>
<tr>
<td>pYG768</td>
<td>pWSK29 with (\text{dinB}^+) under its native promoter</td>
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<td>pYG768(D103N)</td>
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<td>pYG768(F13V)</td>
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<td>pYG768(Y79A)</td>
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<td>pET11T((\text{dinB}^+))</td>
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<td>GCT CGT CAG ACG ATT TAG AGT CTG CAG TG</td>
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References