Environmental and Molecular Mutagenesis 55:92–102 (2014)

Research Article

The DinB•RecA Complex of *Escherichia coli* Mediates an Efficient and High-Fidelity Response to Ubiquitous Alkylation Lesions

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Alkylation DNA lesions are ubiquitous, and result from normal cellular metabolism as well as from treatment with methylating agents and chemotherapeutics. DNA damage tolerance by translesion synthesis DNA polymerases has an important role in cellular resistance to alkylating agents. However, it is not yet known whether *Escherichia coli* (*E. coli*) DNA Pol IV (DinB) alkylation lesion bypass efficiency and fidelity in vitro are similar to those inferred by genetic analyses. We hypothesized that DinB-mediated bypass of 3-deaza-3-methyladenine, a stable analog of 3-methyladenine, the primary replication fork-stalling alkylation lesion, would be of high fidelity. We performed here the first kinetic analyses of *E. coli* DinB•RecA binary complexes. Whether alone or in a binary complex, DinB inserted the correct deoxyribonucleoside triphosphate (dNTP) opposite either lesion-containing or undamaged template; the incorporation of other dNTPs was largely inefficient. DinB prefers undamaged DNA, but the DinB•RecA binary complex increases its catalytic efficiency on lesion-containing template, perhaps as part of a regulatory mechanism to better respond to alkylation damage. Notably, we find that a DinB derivative with enhanced affinity for RecA, either alone or in a binary complex, is less efficient and has a lower fidelity than DinB or DinB•RecA. This finding contrasts our previous genetic analyses. Therefore, mutagenesis resulting from alkylation lesions is likely limited in cells by the activity of DinB•RecA. These two highly conserved proteins play an important role in maintaining genomic stability when cells are faced with ubiquitous DNA damage. Kinetic analyses are important to gain insights into the mechanism(s) regulating TLS DNA polymerases. Environ. Mol. Mutagen. 55:92–102, 2014.

Key words: enzyme kinetics; *Escherichia coli*; mutagenesis; nucleotide; protein complexes; DNA replication

INTRODUCTION

The preservation of genomic integrity is paramount, yet the DNA of all organisms is constantly damaged, and accumulates a variety of lesions. These chemically modified bases are potentially cytotoxic; left unrepaired, they block replication fork progression and cause the accumulation of single-stranded DNA (Friedberg, 2006). Several high-fidelity DNA repair pathways prevent cell death by removing these lesions and restoring the original sequence of the affected DNA. Such pathways include nucleotide excision repair, base excision repair, and homologous recombination (Friedberg, 2006).

Additionally, cells possess enzymes that allow for the tolerance, but not the removal of DNA lesions, which function in pathways that supplement high-fidelity DNA repair processes when the damage is extensive. Translesion synthesis (TLS) utilizes specialized DNA polymerases, which insert nucleotides opposite to and elongate from fork-stalling lesions, thereby restoring normal replication (Ohmori et al., 2001; Friedberg, 2006; Sale et al., 2012). Unlike replicative DNA polymerases, which are responsible for the majority of DNA replication, TLS DNA polymerases lack a proofreading subunit and possess a wider active site; it has been proposed that this wider active site allows for the accommodation of bulky adducts and permits nucleotide incorporation to occur, albeit with the

Grant sponsor: NIGMS; Grant number: 1RO1GM088230-01A1.

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Received 1 August 2013; provisionally accepted 12 September 2013; and in final form 7 October 2013

DOI 10.1002/em.21826

Published online 15 November 2013 in Wiley Online Library (wileyonlinelibrary.com).

potential of a greater frequency of misincorporation (Friedberg et al., 2001; Yang, 2003; Jarosz et al., 2007).

Most organisms typically encode multiple TLS DNA polymerases (Ohmori et al., 2001; Guo et al., 2009; Waters et al., 2009; Andersson et al., 2010). The most conserved of these belongs to the DinB family (Ohmori et al., 2001). In the model system *Escherichia coli* (*E. coli*), the *dinB* gene encodes Y-family DNA Polymerase IV (DinB). Basal levels of *E. coli* DNA Pol IV are relatively high (~250 molecules) (Kim et al., 2001), and this protein is readily upregulated upon DNA damage as part of the SOS response (Friedberg et al., 2006). Upon full SOS induction, DNA Pol IV is the most abundant polymerase in the cell (~2,500 molecules) (Kim et al., 2001), indicating this enzyme plays a pivotal role in the response to DNA damage, as the lesions bypassed by DNA Pol IV are likely to be prevalent in vivo.

Generated by both endogenous and exogenous sources, alkylation damage is ubiquitous. Such damage results not only from treatment with methylating agents, such as methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS) (Beranek, 1990), and with chemotherapeutics (Plosky et al., 2008; Kondo et al., 2010), but also from normal cellular metabolism (De Bont and van Larebeke, 2004). In bacteria, endogenous sources of methylation damage are produced by nitrosation, catalyzed by metabolic enzymes (Taverna and Sedgwick, 1996; Sedgwick,1997). In humans, small reactive molecules such as *S*-adenosylmethionine, betaine, and choline function as endogenous methylating agents [reviewed in De Bont and van Larebeke (2004)]. Furthermore, alkylation yields several types of DNA lesions that vary in abundance. The most frequently produced of these lesions is N^7-methylguanine, which is relatively harmless, as it does not stall replication forks. However, this particular lesion is easily destabilized; cleavage of the glycosyl bond generates abasic sites (Larson et al., 1985; Tudek et al., 1992; De Bont and van Larebeke, 2004). While N^3-methyladenine represents only 10−20% of adducts formed, it is the primary cytotoxic lesion generated by alkylation (Larson et al., 1985; Beranek, 1990; De Bont and van Larebeke, 2004). N^3-methylthymine and N^3-methylcytosine are also formed as a result of alkylation; though potent inhibitors of replication, these lesions are the least abundant (Beranek, 1990; De Bont and van Larebeke, 2004).

Genetic analyses have suggested that DinB-like proteins play a key role in the tolerance of alkylation lesions (Bjedov et al., 2007; Plosky et al., 2008; Benson et al., 2011; Cafarelli et al., 2013). We and others have demonstrated that in *E. coli*, catalytically active DinB is required for resistance to various alkylating agents, such as MMS, EMS, and MNNG (Bjedov et al., 2007; Benson et al., 2011; Cafarelli et al., 2013). Furthermore, low levels of alkylation damage-induced mutagenesis have been measured in $dinB^+$ cells, and most induced mutations depend on other TLS polymerases, particularly *E. coli* Pol V (Bjedov et al., 2007; Benson et al., 2011; Cafarelli et al., 2013). These studies indicate that DinB's activity is required for the bypass of alkylation lesions in vivo; however, these studies do not indicate the efficiency, fidelity, and rate of DinB-mediated bypass. Thus, while there is genetic evidence supporting the role of *E. coli* DinB and its homologs in the bypass of alkylation damage, the catalytic efficiency and fidelity of lesion bypass have not been explored in vitro. Kinetic analyses of *E. coli* DinB alone have been carried out previously; however, these have been limited to undamaged templates (Wagner et al., 1999; Sharma et al., 2013) or templates containing N^2-dG adducts (Jarosz et al., 2006, 2009; Minko et al., 2008; Walsh et al., 2012), or oxidized bases (Hori et al., 2010). Nonetheless, analysis of N^2-dG lesion bypass has allowed us to understand the preference and proficiency of DinB on this lesion, and to ultimately gain insights into the mechanism(s) regulating TLS DNA polymerases.

We reported that the catalytic activity of DinB is modulated by the formation of multi-protein complexes (Godoy et al., 2007), and showed that in vivo, DinB is primarily present in a binary complex with RecA (Cafarelli et al., 2013), the cell's main recombinase. Given the prevalence of alkylation damage, the cytotoxic nature of 3-methyladenine, and the dependence on $dinB^+$ for cellular resistance to alkylating agents, we hypothesized that DinB effects efficient high-fidelity bypass of 3-deaza-3-methyladenine, the stable analog generated by Plosky et al. (2008). To assess the effect of binary complex formation, the most relevant form in vivo, in the tolerance of alkylation lesions, we measured here for the first time the activity and fidelity of DinB•RecA binary complexes. Finally, we utilized the site-specific mutant DinB(C66A), which exhibits an increased affinity for RecA (Cafarelli et al., 2013), to investigate the effect of increased binary complex formation. We find that *E. coli* DinB is catalytically proficient in the bypass of 3-deaza-3-methyladenine. Remarkably, RecA detectably enhances DinB's catalytic activity on lesion-containing template. Moreover, DinB(C66A), which has a higher affinity for RecA than DinB, is proficient for alkylation lesion bypass, whether alone or in complex with RecA, but is less efficient and accurate than native DinB•RecA. These results suggest that protein–protein interactions modulating DinB•RecA complex formation are essential to maintain the full activity of DinB. Therefore, when bound to RecA, *E. coli* DinB plays a direct and essential role in limiting alkylation damage-induced mutagenesis.

MATERIALS AND METHODS

Protein Overexpression and Purification

The *recA* gene, containing an N-terminal hexahistidine tag, was previously cloned in pCA24N (Kitagawa et al., 2005) and was introduced

in BL21 AI △*dinB* △*umuDC* △*recA* cells (Life Technologies) by transformation. His-RecA was overexpressed by autoinduction as previously described (Studier, 2005; Cafarelli et al., 2013), in ZYM-5052 with 20 μg mL^{-1} chloramphenicol. After 48 hr of incubation at 20°C, a cell-free lysate was prepared as previously described (Cafarelli et al., 2013). Lysate containing His-RecA was loaded onto two 1-mL HisTrap HP columns (GE Healthcare Life Sciences; Uppsala, Sweden) with buffer H_A (50 mM HEPES, pH 7.5, 5% glycerol, 0.5 M NaCl, 40 mM imidazole, and 2 mM 2-mercaptoethanol) at a rate of 1 min^{-1}. The column was washed with 8% buffer H_B (50 mM HEPES, pH 7.5, 5% glycerol, 0.5 M NaCl, 0.5 M imidazole, and 2 mM 2-mercaptoethanol), and His-RecA was eluted with a 60% H_B step gradient; the eluted fractions were dialyzed overnight using Spectra/Por (12–14 kDa MWCO) dialysis membrane at 4°C (Spectrum, Laguna Hills, CA) in 150 mM NaCl Tris buffer (20 mM Tris-HCl pH 7.5, 10% glycerol, 2 mM 2-mercaptoethanol). Dialyzed fractions were loaded onto a 5-mL HiTrap Q HP anion exchange column (GE Healthcare Life Sciences), washed with 50 mM NaCl Tris buffer, and eluted with 300 mM NaCl Tris buffer. Eluted fractions were concentrated with an Amicon Ultra 30-kDa MWCO spin column (Millipore, Billerica, MA) and flash frozen. Native DinB and DinB(C66A) were purified as previously described (Cafarelli et al., 2013). Proteins were stored in aliquots at −80°C. Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Thermo Scientific; Rockford, IL), which was used according to the manufacturer's instructions.

Extension Assays

The fluorescently labeled primer (5′-/HEX/CACTGCAGACTCTA AA-3′A) was annealed to undamaged (5′-GCTCGTCAGACG**A**TTTA-GAGTCTGCAGTG-3′) or lesion-containing (5′-GCTCGTCAGACG/**3-deaza-3-methylA**/TTTAGAGTCTGCAGTG) template DNA. Thus, either adenine or 3-deaza-3-methyladenine was present at the primer-template junction. Primer-template was prepared as previously described (Cafarelli et al., 2013). Primer extension reactions were performed in buffer containing 20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg mL^{-1} bovine serum albumin. Reactions containing all of the deoxyribonucleoside triphosphates (dNTPs; Thermo Scientific) had 1.25 μM of DinB or DinB(C66A), 0.25 nM primer-template, and 500 μM dNTP mixture. Equimolar concentrations of RecA were added when needed, and RecA was incubated with DinB or DinB(C66A) prior to addition to the reaction mixtures. Reactions were initiated by addition of enzyme, incubated at 37°C for 10 min, and quenched with 2 μL of stop/loading dye (Ausubel, 2001). Reaction mixtures to determine fidelity of insertion were carried out as indicated above, but included varying concentrations of individual dNTPs (0–1,000 μM; GE Healthcare Life Sciences). Extension products were separated on a 10% denaturing acrylamide gel containing urea and visualized using a Typhoon 8600 Imager. The fluorescence intensity of the unextended primer and the separated extension products was determined using ImageJ software (NIH). Percent total extension was calculated by dividing the intensity of all extension products, including full-length primer and all intermediate products, by the intensity of all detected products. Prism 6 (GraphPad) was used to generate Michaelis–Menten plots and to determine V_{max} and K_m. All extension reactions were performed in duplicate; the results obtained for each replicate were reproducible.

RESULTS

Efficient In Vitro Bypass of 3-Deaza-3-methyladenine by DinB•RecA

We first assessed the ability of DinB, in a binary complex with RecA, to replicate DNA using either an undamaged or alkylation lesion-containing template. The chemical structures of 2′-deoxyadenosine, the normal nucleoside; 2′-deoxy-3′-methyladenine, the fork-stalling alkylation lesion; and 2′-deoxyadenosine-3-deaza-3′-methyladenine, the stable analog generated by Plosky et al. (2008) are shown in Figure 1A. A schematic of the undamaged and 3-deaza-3-methyladenine-containing primer-templates are shown in Figure 1B for reference.

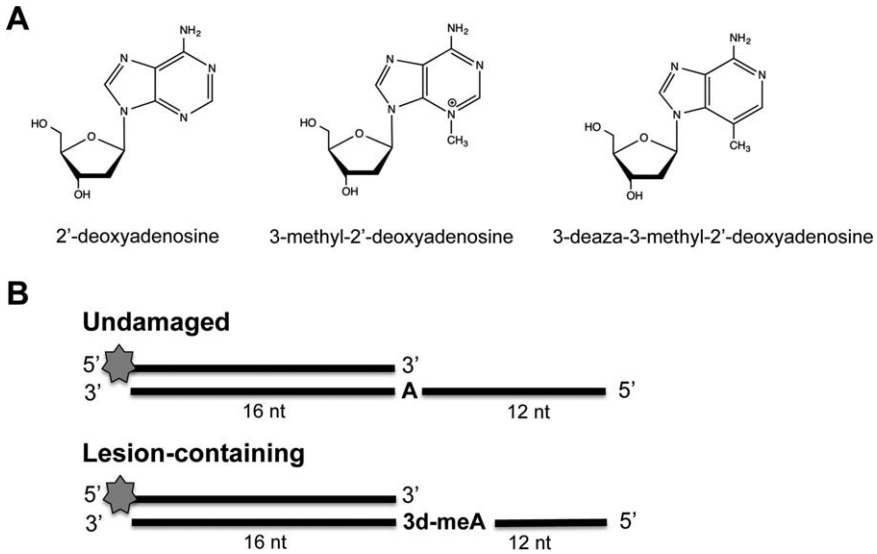

Fig. 1. Structures of alkylation lesions and schematic of primer-templates used for standing-start primer extension assays. (**A**) Chemical structures of the normal nucleoside, 2′-deoxydenosine; the primary replication fork-stalling alkylation lesion, 3-methyl-2′-deoxyadenosine; and its stable analog, 3-deaza-3-methyl-2′-deoxyadenosine. (**B**) Schematic of primer-templates used for standing-start extension assays. Undamaged template contained adenine (A), while lesion-containing template contained 3-deaza-3-methyladenine (3d-meA) at the same position. A common primer was independently annealed to both templates, and contained a hexachlorofluorescein (HEX) label at the 5′ end (indicated by gray star).

As previously reported, DinB efficiently replicates both undamaged and lesion-containing templates (Cafarelli et al., 2013). To better examine the efficiency of bypass, DinB or DinB•RecA was diluted, as indicated in Figure 2, and its catalytic activity was measured. Titration of the enzyme resulted in reduced efficiency of DNA synthesis, as full-length product was detected only at higher DinB or DinB•RecA concentrations (Fig. 2A). As little as 25 nM of enzyme was sufficient to generate full-length product (third lane in each respective panel, Fig. 2A). The fraction of total extension, which takes into consideration full-length product and all shorter intermediates, was comparable for both undamaged and lesion-containing templates (65 and 79%, respectively, for undiluted

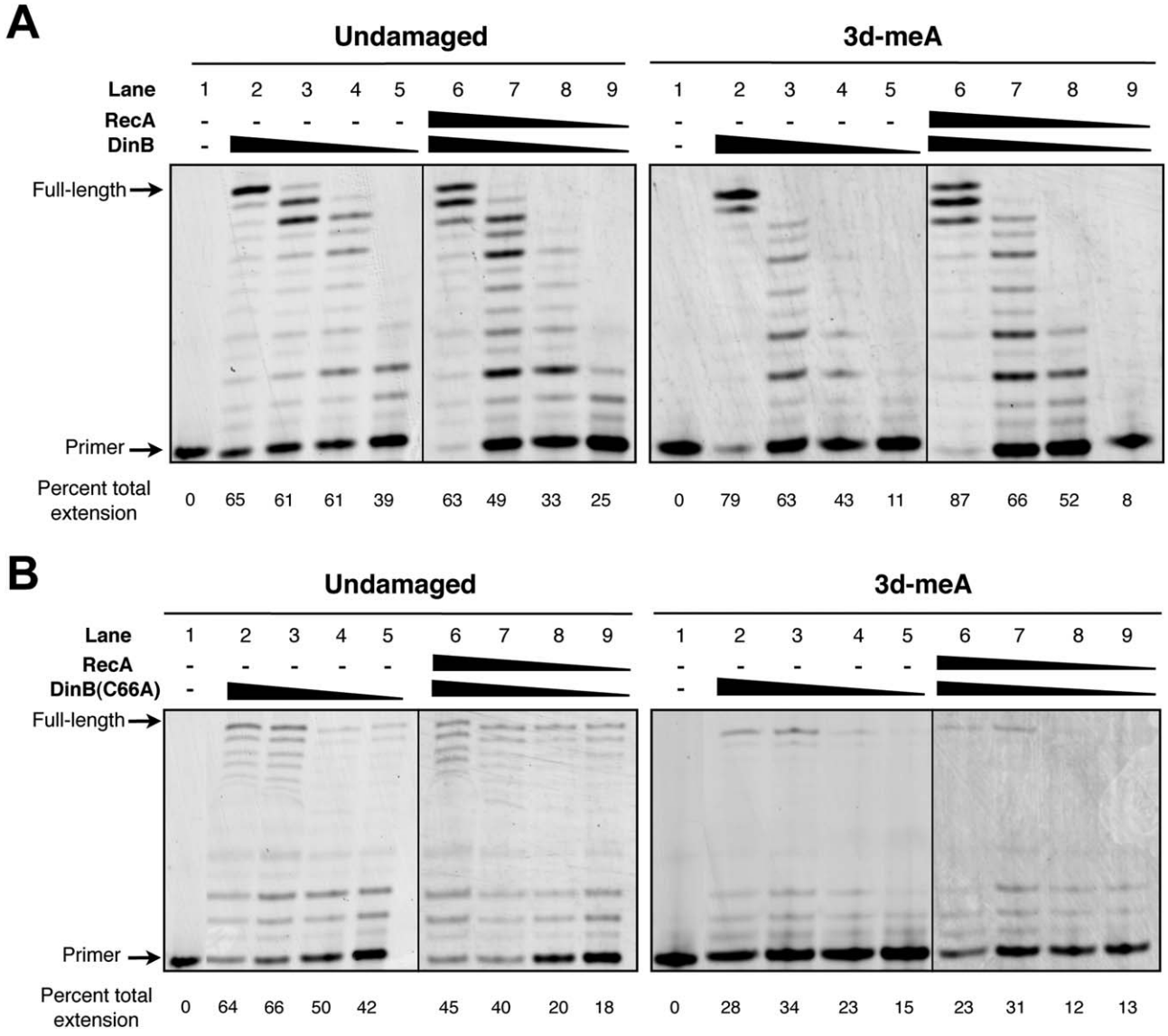

Fig. 2. DinB or DinB(C66A), alone or in a binary complex with RecA, bypasses 3-deaza-3-methyladenine in vitro. (**A**) Bypass by DinB and DinB•RecA. Standing-start primer extension products generated by *E. coli* DinB or DinB•RecA. Undiluted reactions contained 1.25 μM of DinB, or 1.25 μM each of DinB and RecA, as well as 500 μM dNTP mixture. The enzyme alone or enzyme complex was titrated by diluting initial concentrations 1:50, 1:100, and 1:500. Shown are extension products generated on undamaged primer-template (left panel) and primer-template containing 3-deaza-3-methyladenine (right panel). Extension products were separated on a 10% acrylamide gel, visualized using a Typhoon 8600 scanner, and quantified using ImageJ software. The percent total extension was calculated for each reaction, as indicated in Materials and Methods. (**B**) Bypass by DinB(C66A) and DinB(C66A)•RecA. Standing-start primer extension products generated by *E. coli* DinB(C66A) or DinB(C66A)•RecA. Undiluted reactions were as described in (A), but because DinB(C66A) is not as stable as DinB in vitro, the enzyme alone or enzyme complex was titrated by diluting initial concentrations 1:2, 1:5, and 1:10. Shown are extension products generated on undamaged primer-template substrate (left panel) and primer-template substrate containing 3-deaza-3-methyladenine (right panel). Extension products were separated and quantified as described previously. Reactions were performed in duplicate, and results were consistent between replicates.

enzyme; lane 2 of each panel, Fig. 2A). The activity of the DinB•RecA complex is similar to that of DinB (Fig. 2A), as the amount of full-length product and intermediate products was not substantially decreased in the presence of RecA (lanes 6–9; Fig. 2A).

Proficient Replication of Alkylation Lesion-Containing Template by the DinB(C66A)•RecA Binary Complex

We have recently reported that DinB(C66A) is catalytically active, has a higher binding affinity for RecA than DinB, and causes a decrease in the frequency of DNA damage-induced mutagenesis when expressed from the chromosome in vivo (Cafarelli et al., 2013). We predicted that DinB(C66A) should therefore effect high-fidelity bypass of alkylating lesions, particularly when in a complex with RecA.

We assessed the proficiency of DinB(C66A) and DinB(C66A)•RecA binary complexes in the replication of undamaged and lesion-containing templates (Fig. 2B). We found that DinB(C66A) generated less full-length product than native DinB when undamaged DNA is used as template (compare lanes 2 of Figs. 2A and 2B). Nevertheless, when percent total extension was calculated, we found that the proficiency of DinB(C66A) was comparable to that detected for native DinB (lanes 2 of Figs. 2B and 2A). These data indicate that, while DinB(C66A) is less efficient in the generation of full-length product, it is able to synthesize undamaged DNA and gave rise to shorter intermediate products.

While DinB displayed robust catalytic activity on lesion-containing template, DinB(C66A) displayed a marked decrease in the ability to generate both full-length product and shorter intermediate products (3dmeA panels of Fig. 2B). It is also critical to note that while the native enzyme was active even when low concentrations were used (12.5 to 25 nM; lanes 4 and 3, respectively, of Fig. 2A), DinB(C66A) began to lose activity at 125–250 nM (lanes 5 and 4, respectively, of each panel; Fig. 2B). While DinB(C66A) is catalytically proficient, it is less efficient than DinB in the replication of both undamaged and lesion-containing DNA. RecA appeared to have very little effect on the catalytic activity of DinB(C66A) on undamaged or lesion-containing template (Fig. 2B).

Accurate Bypass of 3-Deaza-3-methyladenine Catalyzed by the DinB•RecA Binary Complex

We then sought to determine the fidelity of insertion, and to elucidate the degree to which fidelity is altered by protein-protein interactions with RecA. We used the same reaction conditions as those shown in Figure 2, except that individual dNTPs were added (Fig. 3) instead of the dNTP mixture.

We find that DinB preferentially inserted the correct nucleotide (dTTP) opposite the template adenine ($V_{max}/K_m = 6.7 \times 10^9$ fmol min^{-1} mg^{-1} M^{-1}; Table I). Reactions containing dTTP with an undamaged template were particularly efficient since dTTP (Fig. 3B) was inserted approximately 900-fold better than dCTP or 1,000-fold better than dGTP (Figs. 3D and 3C, respectively). Insertion of dATP was detectable (Fig. 3A), but was approximately 2,600-fold less efficient than dTTP insertion (Fig. 3B), and considerably less efficient than dGTP or dCTP (Figs. 3C and 3D, respectively).

The addition of RecA did not alter DinB's activity on undamaged template, as the V_{max}/K_m for dTTP insertion was comparable to that observed for DinB alone (5.0×10^9 fmol min^{-1} mg^{-1} M^{-1} for DinB•RecA; Table I). Furthermore, the fidelity of DinB-mediated insertion was not affected by the addition of RecA. Insertion of dCTP or dATP by DinB•RecA opposite adenine on the undamaged template was largely inefficient, as was seen for DinB alone (900-fold and 2,000-fold less likely than dTTP insertion on undamaged DNA, respectively; Figs. 3D and 3A and Table I). The ability of DinB to incorporate dGTP opposite adenine was decreased when in a complex with RecA (2,600-fold less likely than dTTP insertion; Fig. 3C and Table I). Overall, DNA synthesis by DinB•RecA on an undamaged template was highly accurate.

DinB exhibited a consistent (2-fold) decrease in catalytic activity when replicating 3-deaza-3-methyladenine-containing template, relative to its activity on undamaged template ($V_{max}/K_m = 2.9 \times 10^9$ fmol min^{-1} mg^{-1} M^{-1} for dTTP insertion on lesion-containing DNA, versus 6.7×10^9 fmol min^{-1} mg^{-1} M^{-1} for undamaged template; Fig. 3B and Table I). This indicates that the enzyme alone prefers to replicate undamaged template. The misincorporation of other dNTPs, and the concomitant generation of mismatches, was more likely on 3-deaza-3-methyladenine-containing templates than on undamaged templates (Figs. 3A, 3C, and 3D; Table I).

When in a binary complex with RecA, DinB displayed an enhanced ability to accurately replicate lesion-containing DNA ($V_{max}/K_m = 6.6 \times 10^9$ fmol min^{-1} mg^{-1} M^{-1} for dTTP insertion on lesion-containing template; Fig. 3B and Table I). Additionally, DinB's fidelity was enhanced when bound to RecA. The incorporation of dTTP by DinB•RecA binary complexes is 450-fold more likely dGTP insertion, 800-fold more likely than dCTP insertion, and 3,000-fold more likely than dATP insertion, based on V_{max}/K_m ratios (Table I).

An example of the insertion profiles of DinB and DinB•RecA is shown for each nucleotide in Figure 3E. In most cases, we were able to detect single nucleotide insertions, directly opposite either adenine (undamaged substrate) or 3-deaza-3-methyladenine (lesion-containing substrate). Incorporation of dCTP required a frameshift

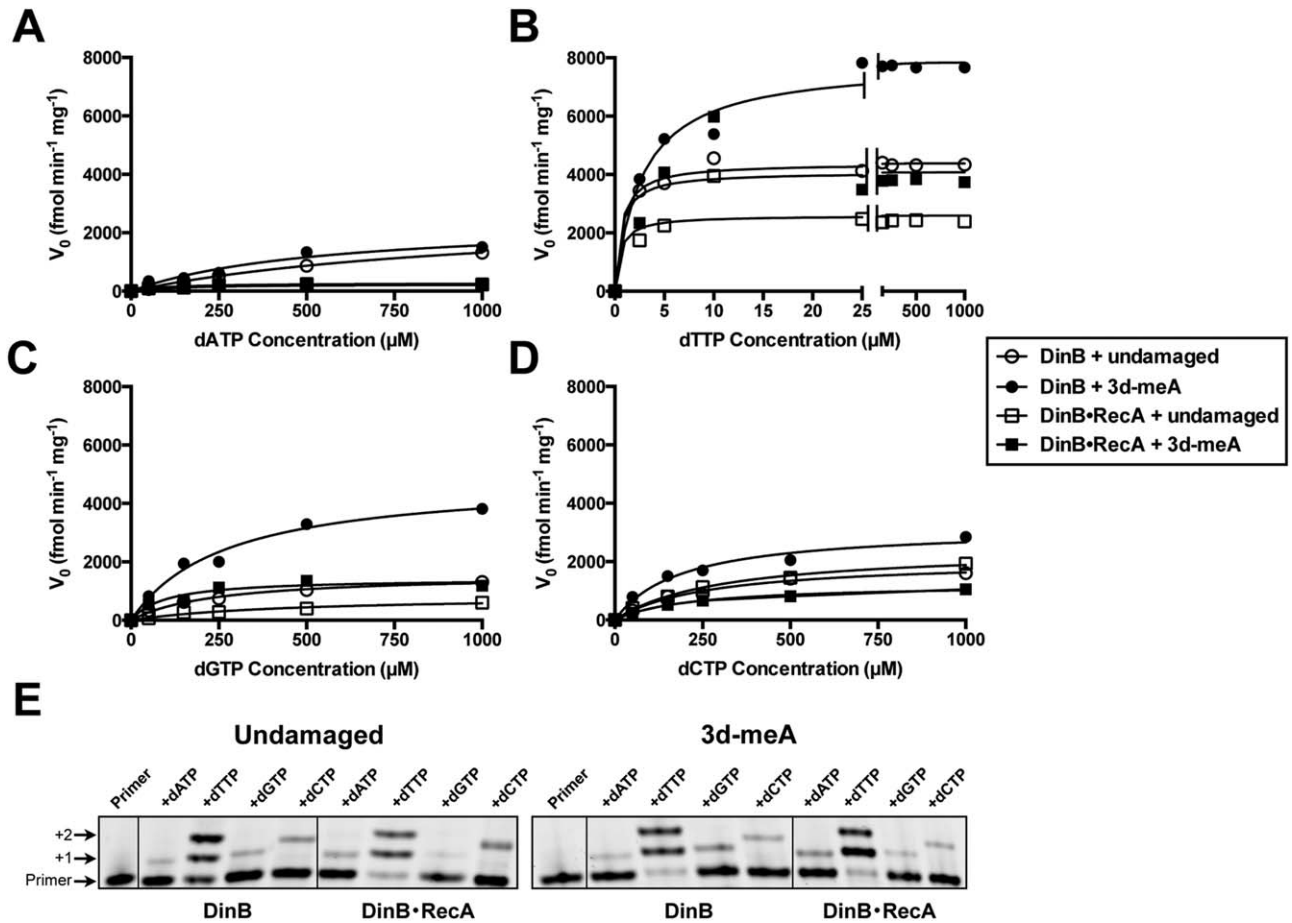

Fig. 3. *In vitro* analyses of the fidelity of DinB and DinB•RecA binary complexes. DinB-catalyzed bypass of 3-deaza-3-methyladenine is accurate, and its fidelity is increased by the formation of DinB•RecA binary complexes. Michaelis-Menten kinetic curves for individual dNTP insertion by DinB and DinB•RecA on undamaged and lesion-containing templates. Insertion kinetic graphs for dATP (**A**), dTTP (**B**), dGTP (**C**), and dCTP (**D**) are shown. DinB and DinB•RecA binary complexes preferentially insert dTTP opposite either adenine (undamaged primer-template) or 3-deaza-3-methyladenine (lesion-containing primer-template). The binding of RecA limits DinB's ability to misincorporate on either template. Insertion profile of each dNTP is shown in (**E**). Reactions shown in (E) contained 1.25 μM of each protein, as indicated, as well as 250 μM of the indicated dNTP. Reactions were performed in duplicate, and results were consistent between replicates.

TABLE I. Summary of Kinetic Parameters of DinB and DinB•RecA Insertion Opposite Adenine or 3-Deaza-3-methyladenine

Polymerase	Template	Incoming nucleotide	V_{max} (fmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/Km (fmol min^{-1} mg^{-1} M^{-1})	Efficiency of insertion
DinB	Undamaged	dTTP	4,381 ± 99	0.65 ± 0.21	6.7×10^9	1.0000
		dCTP	2,066 ± 249	270 ± 85	7.7×10^6	0.0011
		dGTP	1,587 ± 230	245 ± 95	6.5×10^6	0.0010
		dATP	2,722 ± 403	1063 ± 257	2.6×10^6	0.0004
	3d-meA	dTTP	7,856 ± 192	2.75 ± 0.45	2.9×10^9	1.0000
		dGTP	4,860 ± 616	269 ± 89	1.8×10^7	0.0063
		dCTP	3,162 ± 536	190 ± 95	1.7×10^7	0.0058
		dATP	2,424 ± 671	543 ± 305	4.5×10^6	0.0016
DinB•RecA	Undamaged	dTTP	2,589 ± 229	0.52 ± 0.74	5.0×10^9	1.0000
		dCTP	1,237 ± 423	218 ± 209	5.7×10^6	0.0011
		dATP	272 ± 33	113 ± 49	2.4×10^6	0.0005
		dGTP	842 ± 181	448 ± 209	1.9×10^6	0.0004
	3d-meA	dTTP	4,077 ± 332	0.62 ± 0.72	6.6×10^9	1.0000
		dGTP	1,432 ± 181	98 ± 47	1.5×10^7	0.0022
		dCTP	2,451 ± 802	302 ± 246	8.1×10^6	0.0012
		dATP	261 ± 98	119 ± 158	2.2×10^6	0.0003

Calculated values ±1 SD are shown.

event that eliminated the requirement for direct lesion bypass, as the nucleotide immediately after the adenine or 3-deaza-3-methyladenine in the template is a G (Fig. 3E).

Less Efficient, More Error-prone Bypass of 3-Deaza-3-methyladenine by DinB(C66A)•RecA, Compared to DinB•RecA

As was seen for native DinB, DinB(C66A) and DinB (C66A)•RecA preferentially incorporated dTTP on undamaged templates (Fig. 4B; V_{max}/K_m of 6.5×10^7 fmol min^{-1} mg^{-1} M^{-1} for DinB(C66A) and 4.3×10^8 fmol min^{-1} mg^{-1} M^{-1} for DinB(C66A)•RecA; Table II). Surprisingly, DinB(C66A) was able to misincorporate dNTPs at a greater frequency than the wild-type enzyme. Additionally, the catalytic signature of the mutant was quite different. While DinB was least likely to misincorporate dATP, DinB(C66A) was most likely to utilize this nucleotide after dTTP (Fig. 4A). Indeed, the incorporation of dTTP by DinB(C66A) was only 7-fold more efficient than dATP incorporation on undamaged template (Table II). Incorporation of dCTP was also detected (Fig. 4D), albeit at a reduced efficiency relative to the native enzyme, while incorporation of dGTP was largely inefficient and undetectable (Fig. 4C).

The addition of RecA resulted in increased catalytic ability on undamaged DNA; DinB(C66A)•RecA binary complexes exhibited a nearly 7-fold increase in the efficiency of dTTP incorporation (Fig. 4B and Table II). The ability of DinB(C66A)•RecA to misincorporate dATP was also enhanced, but the ability to utilize dGTP or dCTP remained limited.

When lesion-containing template was used, we find that DinB(C66A) had a reduced efficiency of dTTP incorporation, relative to undamaged template (V_{max}/K_m of 4.2×10^7 fmol min^{-1} mg^{-1} M^{-1} vs. 6.5×10^7 fmol min^{-1}

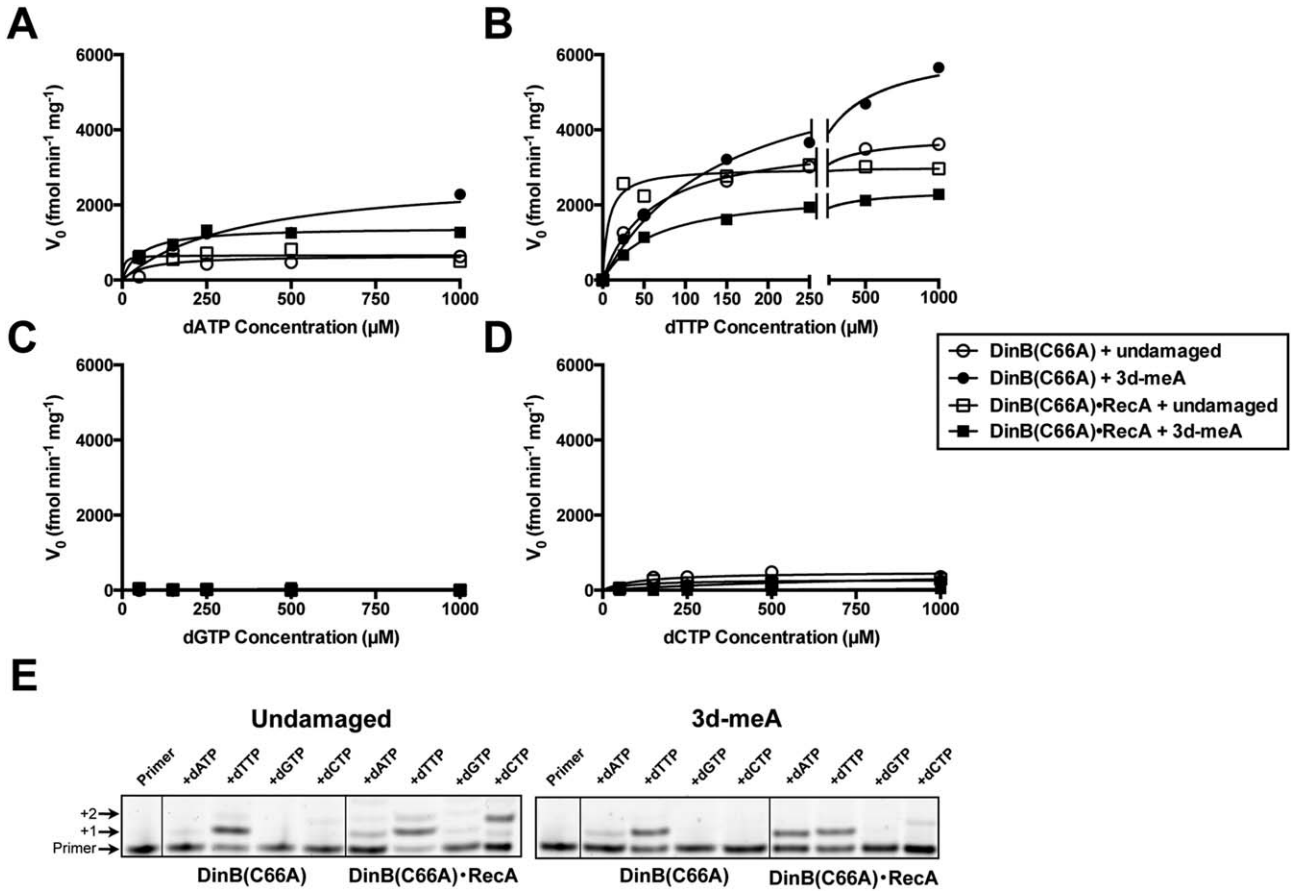

Fig. 4. *In vitro* analyses of the fidelity of DinB(C66A) and DinB (C66A)•RecA binary complexes. DinB(C66A)-catalyzed bypass of 3-deaza-3-methyladenine is accurate, but is less efficient than DinB-mediated insertion. Michaelis-Menten kinetic curves for individual dNTP insertion by DinB(C66A) and DinB(C66A)•RecA on undamaged and lesion-containing templates. Insertion kinetic graphs for dATP (**A**), dTTP (**B**), dGTP (**C**), and dCTP (**D**) are shown. DinB and DinB•RecA binary complexes preferentially insert dTTP opposite either adenine (undamaged primer-template) or 3-deaza-3-methyladenine (lesion-containing primer-template). The addition of RecA enhances dTTP incorporation on undamaged template, but limits DinB(C66A)'s ability to insert dTTP on lesion-containing template. Insertion profile of each dNTP is shown in (**E**). Reactions shown in (E) contained 1.25 μM of each protein, as indicated, as well as 250 μM of the indicated dNTP. Reactions were performed in duplicate, and results were consistent between replicates.

TABLE II. Summary of Kinetic Parameters of DinB(C66A) and DinB(C66A)•RecA Insertion Opposite Adenine or 3-Deaza-3-methyladenine

Polymerase	Template	Incoming nucleotide	V_{max} (fmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/Km (fmol min^{-1} mg^{-1} M^{-1})	Efficiency of insertion
DinB(C66A)	Undamaged	dTTP	3,808 ± 256	59 ± 16	6.5×10^7	1.0000
		dATP	658 ± 163	70 ± 76	9.5×10^6	0.1461
		dCTP	492 ± 116	113 ± 96	4.4×10^7	0.0672
		dGTP	4.2 ± 4.1	76 ± 319	5.5×10^4	0.0008
	3d-meA	dTTP	6,251 ± 465	148 ± 34	4.2×10^7	1.0000
		dATP	2,819 ± 427	355 ± 126	7.9×10^6	0.1873
		dCTP	571 ± 300	953 ± 846	6.0×10^5	0.0141
		dGTP	ND	ND	ND	ND
DinB(C66A)•RecA	Undamaged	dTTP	2,983 ± 178	6.9 ± 4.5	4.3×10^8	1.0000
		dATP	659 ± 178	4.2 ± 31.5	1.6×10^8	0.3619
		dCTP	281 ± 116	90 ± 136	3.1×10^6	0.0072
		dGTP	ND	ND	ND	ND
	3d-meA	dTTP	2,394 ± 103	62 ± 11	3.9×10^7	1.0000
		dATP	1,404 ± 154	55 ± 29	2.6×10^7	0.6680
		dGTP	35 ± 25	64 ± 207	5.5×10^5	0.0141
		dCTP	ND	ND	ND	ND

Calculated values ±1 SD are shown.
ND, not determined, due to low efficiency.

mg^{-1} M^{-1}; Table II). DinB(C66A) was less likely to misincorporate dGTP and dCTP on lesion-containing substrate than on undamaged DNA, but more likely to insert dATP (Table II). Notably, the fidelity of DinB(C66A) on lesion-containing template was decreased when in a binary complex with RecA. The insertion of dTTP was now only 1.5-fold more likely than dATP misincorporation on 3-deaza-3-methyladenine-containing substrates (Fig. 4A and Table II). The ability to utilize dGTP and dCTP remained largely inefficient (Figs. 4C and 4D).

The incorporation profile of individual nucleotides is shown for DinB(C66A) and DinB(C66A)•RecA in Figure 4E. As observed for the native enzyme, most reactions resulted in single nucleotide insertions on either template. The incorporation of dTTP was visibly less efficient than detected for native DinB (Figs. 4E and 3E, respectively), as the second dTTP insertion was limited. The insertion of other dNTPs was also visibly reduced for DinB(C66A), particularly on lesion-containing templates (Fig. 4E).

DISCUSSION

Though a relatively small minor groove DNA lesion (Fig. 1A), 3-methyladenine blocks replication by high-fidelity DNA polymerases (Larson et al., 1985; Johnson et al., 2007). It has been extensively demonstrated by genetic analyses that Y-family translesion DNA polymerases are required for resistance to alkylating agents (Bjedov et al., 2007; Johnson et al., 2007; Plosky et al., 2008; Benson et al., 2011; Cafarelli et al., 2013). It is clear that *E. coli* DinB promotes survival in a high-fidelity manner (Bjedov et al., 2007; Benson et al., 2011; Cafarelli et al., 2013), in contrast to the activity of DNA Pol V ($UmuD'_2C$), which yields high levels of alkylation-induced mutagenesis. However, it is not yet known whether *E. coli* DNA Pol IV (DinB) alkylation lesion bypass efficiency and fidelity in vitro are similar to those inferred by genetic analyses. Moreover, genetic studies alone do not permit us to separate the functions of DinB activity, either alone or in a multi-protein complex (MPC) (Godoy et al., 2007; Cafarelli et al., 2013), because of the key role of RecA, one of the DinB MPC components, in DNA damage signaling (Sutton et al., 2000; Friedberg, 2006). Therefore, in vitro kinetic analyses are a good approach to directly determine the efficiency and fidelity of DinB-mediated bypass of alkylation damage, and to decipher the degree to which DinB's translesion activity and fidelity is altered by multi-protein complex formation.

In this report, we find that DinB proficiently and accurately bypasses 3-deaza-3-methyladenine, the stable analog of 3-methyladenine generated by Plosky et al. (2008). DinB preferentially inserts cognate dTTP in the standing-start primer extension assay that was performed to measure DinB's efficiency and fidelity (Fig. 3). Notably, we find that the efficiency of dTTP incorporation by DinB was 2-fold greater for the undamaged template than the lesion-containing template, primarily as a result of an increased K_m for dTTP on lesion-containing DNA (Table I and Fig. 3). This is in contrast with previous studies, in which a preference for lesion-containing template was observed in the bypass of N_2-furfuryl-dG, another DinB-cognate lesion (Jarosz et al., 2006, 2009). Jarosz et al. (2006) reported that insertion of dCTP was about 16-fold more efficient on templates containing N_2-furfuryl-dG

than on undamaged templates. Thus, our results indicate that, while both N_2-furfuryl-dG and N_3-methyladenine may be DinB-cognate lesions, N_2-furfuryl-dG is likely to be the preferred lesion.

In *E. coli*, DinB's activity is regulated post-translationally by the formation of stable binary and ternary complexes (Godoy et al., 2007). Interaction with RecA, the cell's main recombinase, and UmuD, an accessory subunit, visibly alters the catalytic ability of DinB; the ability to generate -1 frameshifts is reduced, while the ability to catalyze undamaged DNA is enhanced (Godoy et al., 2007). We recently presented evidence suggesting that DinB is primarily available in vivo in a binary complex with RecA (Cafarelli et al., 2013). Given the importance of protein-protein interactions in the regulation of DinB's activity, and the proposed abundance of DinB•RecA binary complexes, we sought to describe the effect of RecA binding on the alkylation lesion bypass mediated by DinB. We therefore describe for the first time the effect of binary complex formation on the TLS activity of DinB. We find that, in accordance with Godoy et al. (2007), the catalytic ability of DinB was enhanced in the presence of RecA; DinB•RecA binary complexes have greater catalytic efficiency than DinB when inserting dTTP opposite 3-deaza-3-methyladenine. The ability of RecA to regulate the activity of other DNA polymerases has been previously described (Schlacher et al., 2005; Jiang et al., 2009; Karata et al., 2012; Pages et al., 2012; Indiani et al., 2013), and it has been postulated that RecA may regulate the occupancy of various replicative and TLS polymerases at the replication fork (Indiani et al., 2013). In fact, it has been shown that TLS is substantially limited in vivo in the absence of RecA (Pages et al., 2012). Intriguingly, however, we observed an increase in catalytic activity only in the replication of lesion-containing template. RecA appeared to have no effect on the efficiency of DinB in the replication of undamaged template. The ability of RecA to enhance the catalytic activity of DinB may occur as part of a regulatory mechanism, which allows cells to better respond to persistent and ubiquitous DNA lesions, such as those generated by alkylation. Indeed, RecA and DinB might function in concert to limit alkylation damage-induced mutagenesis in vivo.

The data presented here are consistent with the kinetic data observed for Pol Kappa, the eukaryotic homolog of DinB. Like Pol Kappa, DinB displays an enhanced ability to insert on undamaged DNA, relative to lesion-containing DNA [2-fold; Fig. 3 and Table I; (Plosky et al., 2008)]. We also find that the fidelity of DinB-mediated bypass is greater than the reported fidelity of human Pol Kappa. Pol Kappa was only 8 to 25-fold more likely to incorporate dTTP on lesion-containing template (Plosky et al., 2008). In contrast, DinB is much more likely to insert the correct dNTP than to misincorporate (Fig. 3 and Table I). This agrees with data collected by Bjedov et al. (2007), which suggests that the majority of alkylation-induced mutagenesis detected in vivo is effected by other polymerases in *E. coli* (e.g., Pol V). Indeed, *E. coli* likely utilizes DinB for the efficient and accurate bypass of alkylation lesions. Given the enhanced fidelity of DinB, it appears as though the bacterial enzyme is particularly adept at dealing with alkylation damage. This is also consistent with our previous findings (Benson et al., 2011).

The DinB(C66A) derivative was recently characterized, and like the native enzyme, was found to be catalytically active and proficient for bypass of 3-deaza-3-methyladenine (Cafarelli et al., 2013). This enzyme was used for comparative purposes in these analyses because we know that *dinB(C66A)* cells are less mutagenic than *dinB*$^+$ (Cafarelli et al., 2013). Like native DinB, DinB(C66A) is more efficient on undamaged than on damaged templates, particularly when bound to RecA. However, in vitro characterization reveals that this derivative is considerably less efficient than the native enzyme (Fig. 2B); this may be due, at least in part, to reduced stability of DinB(C66A), relative to DinB. However, it is unclear whether the observed decrease in bypass efficiency is the result of decreased DNA recognition and binding, decreased translesion bypass ability, or a combination of both. Additionally, DinB(C66A) was found to have a larger K_m for dTTP in the presence of either template (Table II), indicating that the ability to utilize specific nucleotides may be reduced relative to DinB. The fidelity of DinB(C66A) was also reduced relative to DinB, as this derivative was more likely to misincorporate dNTPs on either template than the native protein. In fact, DinB(C66A) has a distinct catalytic signature. The order of insertion preference detected for DinB was dTTP>> dCTP or dGTP> dATP (Table I), but was dTTP>dATP>dCTP>dGTP for DinB(C66A) (Table II). However, given that DinB(C66A) still preferentially utilizes dTTP in the bypass of 3-deaza-3-methyladenine and that dTTP is the most abundant dNTP in *E. coli* (Buckstein et al., 2008), it is likely that even this derivative performs accurate, albeit possibly slower, translesion bypass of such lesions in vivo.

While all organisms must contend with ubiquitous alkylation damage, the mutagenic potential of alkylation lesions varies considerably between prokaryotes and eukaryotes. In eukaryotic systems, multiple polymerases, including Pol Kappa, function in concert to resolve alkylation-induced lesions. *E. coli* appears to rely primarily on DinB to accurately and efficiently bypass such lesions. Cells containing functional DinB are able to limit the mutagenic potential of alkylation lesions. The present study is therefore consistent with the absence of mutations in DinB in naturally occurring bacterial isolates (Bjedov et al., 2003; Benson et al., 2011), and in Pol

Kappa in sequenced human genomes. We show here that mutations, even those that do not target the catalytic core of the enzyme [for example, DinB(C66A)], may alter DinB's inherent fidelity. It is also likely that, while the polymerase itself does not tolerate mutations, its interacting proteins might be more variable, which in turn may affect DinB's fidelity. Understanding these enzymes and their interplay with interacting proteins will permit us to understand how cells maintain genomic stability in an ever-changing environment.

ACKNOWLEDGEMENTS

This work was funded by the 1RO1GM088230-01A1 award from NIGMS to V. G. Godoy. The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

T.M. Cafarelli and V.G. Godoy designed experiments, analyzed the data, and composed the manuscript. T. M. Cafarelli and T. J. Rands performed experiments. All authors approved the manuscript draft.

REFERENCES

Andersson DI, Koskiniemi S, Hughes D. 2010. Biological roles of translesion synthesis DNA polymerases in eubacteria. Mol Microbiol 77:540–548.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 2001. Current protocols in Molecular Biology. New York: Wiley.

Benson RW, Norton MD, Lin I, Du Comb WS, Godoy VG. 2011. An active site aromatic triad in *Escherichia coli* DNA Pol IV coordinates cell survival and mutagenesis in different DNA damaging agents. PLoS One 6:e19944.

Beranek DT. 1990. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat Res 231: 11–30.

Bjedov I, Dasgupta CN, Slade D, Le Blastier S, Selva M, Matic I. 2007. Involvement of *Escherichia coli* DNA polymerase IV in tolerance of cytotoxic alkylating DNA lesions in vivo. Genetics 176:1431–1440.

Bjedov I, Lecointre G, Tenaillon O, Vaury C, Radman M, Taddei F, Denamur E, Matic I. 2003. Polymorphism of genes encoding SOS polymerases in natural populations of *Escherichia coli*. DNA Repair (Amst) 2:417–426.

Buckstein MH, He J, Rubin H. 2008. Characterization of nucleotide pools as a function of physiological state in *Escherichia coli*. J Bacteriol. 190:718–726.

Cafarelli TM, Rands TJ, Benson RW, Rudnicki PA, Lin I, Godoy VG. 2013. A single residue unique to DinB-like proteins limits formation of the Pol IV multi-protein complex in *Escherichia coli*. J Bacteriol. 195:1179–1193.

De Bont R, van Larebeke N. 2004. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19:169–185.

Friedberg EC, Fischhaber PL, Kisker C. 2001. Error-prone DNA polymerases: novel structures and the benefits of infidelity. Cell 107:9–12.

Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA, Ellenberger T. 2006. DNA Repair and Mutagenesis, 2nd ed. Washington, DC: ASM Press. xxix, 1118 p., [1111] p. of plates p.

Godoy VG, Jarosz DF, Simon SM, Abyzov A, Ilyin V, Walker GC. 2007. UmuD and RecA directly modulate the mutagenic potential of the Y family DNA polymerase DinB. Mol Cell 28:1058–1070.

Guo C, Kosarek-Stancel JN, Tang TS, Friedberg EC. 2009. Y-family DNA polymerases in mammalian cells. Cell Mol Life Sci 66: 2363–2381.

Hori M, Yonekura S, Nohmi T, Gruz P, Sugiyama H, Yonei S, Zhang-Akiyama QM. 2010. Error-Prone Translesion DNA Synthesis by *Escherichia coli* DNA Polymerase IV (DinB) on Templates Containing 1,2-dihydro-2-oxoadenine. J Nucleic Acids 2010:807579.

Indiani C, Patel M, Goodman MF, O′Donnell ME. 2013. RecA acts as a switch to regulate polymerase occupancy in a moving replication fork. Proc Natl Acad Sci USA 110:5410–5415.

Jarosz DF, Beuning PJ, Cohen SE, Walker GC. 2007. Y-family DNA polymerases in *Escherichia coli*. Trends Microbiol 15:70–77.

Jarosz DF, Cohen SE, Delaney JC, Essigmann JM, Walker GC. 2009. A DinB variant reveals diverse physiological consequences of incomplete TLS extension by a Y-family DNA polymerase. Proc Natl Acad Sci USA 106:21137–21142.

Jarosz DF, Godoy VG, Delaney JC, Essigmann JM, Walker GC. 2006. A single amino acid governs enhanced activity of DinB DNA polymerases on damaged templates. Nature 439:225–228.

Jiang Q, Karata K, Woodgate R, Cox MM, Goodman MF. 2009. The active form of DNA polymerase V is UmuD′(2)C-RecA-ATP. Nature 460:359–363.

Johnson RE, Yu SL, Prakash S, Prakash L. 2007. A role for yeast and human translesion synthesis DNA polymerases in promoting replication through 3-methyl adenine. Mol Cell Biol 27:7198–7205.

Karata K, Vaisman A, Goodman MF, Woodgate R. 2012. Simple and efficient purification of *Escherichia coli* DNA polymerase V: cofactor requirements for optimal activity and processivity in vitro. DNA Repair (Amst) 11:431–440.

Kim SR, Matsui K, Yamada M, Gruz P, Nohmi T. 2001. Roles of chromosomal and episomal *dinB* genes encoding DNA pol IV in targeted and untargeted mutagenesis in *Escherichia coli*. Mol Genet Genomics 266:207–215.

Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H. 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. DNA Res 12:291–299.

Kondo N, Takahashi A, Ono K, Ohnishi T. 2010. DNA damage induced by alkylating agents and repair pathways. J Nucleic Acids 2010: 543531.

Larson K, Sahm J, Shenkar R, Strauss B. 1985. Methylation-induced blocks to in vitro DNA replication. Mutat Res 150:77–84.

Minko IG, Yamanaka K, Kozekov ID, Kozekova A, Indiani C, O′Donnell ME, Jiang Q, Goodman MF, Rizzo CJ, Lloyd RS. 2008. Replication bypass of the acrolein-mediated deoxyguanine DNA-peptide cross-links by DNA polymerases of the DinB family. Chem Res Toxicol 21:1983–1990.

Ohmori H, Friedberg EC, Fuchs RP, Goodman MF, Hanaoka F, Hinkle D, Kunkel TA, Lawrence CW, Livneh Z, Nohmi T, Prakash L, Prakash S, Todo T, Walker GC, Wang Z, Woodgate R. 2001. The Y-family of DNA polymerases. Mol Cell 8:7–8.

Pages V, Mazon G, Naiman K, Philippin G, Fuchs RP. 2012. Monitoring bypass of single replication-blocking lesions by damage avoidance in the *Escherichia coli* chromosome. Nucleic Acids Res 40:9036–9043.

Plosky BS, Frank EG, Berry DA, Vennall GP, McDonald JP, Woodgate R. 2008. Eukaryotic Y-family polymerases bypass a 3-methyl-2′-deoxyadenosine analog in vitro and methyl methanesulfonate-induced DNA damage *in vivo*. Nucleic Acids Res 36:2152–2162.

Sale JE, Lehmann AR, Woodgate R. 2012. Y-family DNA polymerases and their role in tolerance of cellular DNA damage. Nat Rev Mol Cell Biol 13:141–152.

Schlacher K, Leslie K, Wyman C, Woodgate R, Cox MM, Goodman MF. 2005. DNA polymerase V and RecA protein, a minimal mutasome. Mol Cell 17:561–572.

Sedgwick B. 1997. Nitrosated peptides and polyamines as endogenous mutagens in O6-alkylguanine-DNA alkyltransferase deficient cells. Carcinogenesis 18:1561–1567.

Sharma A, Kottur J, Narayanan N, Nair DT. 2013. A strategically located serine residue is critical for the mutator activity of DNA polymerase IV from *Escherichia coli*. Nucleic Acids Res 41:5104–5114.

Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41:207–234.

Sutton MD, Smith BT, Godoy VG, Walker GC. 2000. The SOS response: recent insights into *umuDC*-dependent mutagenesis and DNA damage tolerance. Annu Rev Genet 34:479–497.

Taverna P, Sedgwick B. 1996. Generation of an endogenous DNA-methylating agent by nitrosation in *Escherichia coli*. J Bacteriol 178:5105–5111.

Tudek B, Boiteux S, Laval J. 1992. Biological properties of imidazole ring-opened N7-methylguanine in M13mp18 phage DNA. Nucleic Acids Res 20:3079–3084.

Wagner J, Gruz P, Kim SR, Yamada M, Matsui K, Fuchs RP, Nohmi T. 1999. The *dinB* gene encodes a novel *E. coli* DNA polymerase, DNA pol IV, involved in mutagenesis. Mol Cell 4:281–286.

Walsh JM, Parasuram R, Rajput PR, Rozners E, Ondrechen MJ, Beuning PJ. 2012. Effects of non-catalytic, distal amino acid residues on activity of *E. coli* DinB (DNA polymerase IV). Environ Mol Mutagen 53:766–776.

Waters LS, Minesinger BK, Wiltrout ME, D′Souza S, Woodruff RV, Walker GC. 2009. Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. Microbiol Mol Biol Rev 73:134–154.

Yang W. 2003. Damage repair DNA polymerases Y. Curr Opin Struct Biol 13:23–30.

Accepted by—
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