Residues in the fingers domain of the translesion DNA polymerase DinB enable its unique participation in error-prone double strand break repair

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Running Title: DinB fingers residues mediate strand displacement

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ABSTRACT

The evolutionarily conserved Escherichia coli translesion DNA Polymerase IV (DinB)¹ is one of three enzymes that can bypass potentially deadly DNA lesions on the template strand during DNA replication. Remarkably, however, DinB is the only known translesion DNA polymerase active in RecA-mediated strand exchange during error-prone double strand break repair. In this process, a ssDNA-RecA nucleoprotein filament invades homologous dsDNA, pairing the ssDNA with the complementary strand in the dsDNA. When exchange reaches the 3’ end of the ssDNA, a DNA polymerase can add nucleotides onto the end, using one strand of dsDNA as a template and displacing the other. It is unknown what makes DinB uniquely capable of participating in this reaction. To explore this topic, we performed molecular modelling of DinB’s interactions with the RecA filament during strand exchange, identifying key contacts made with residues in the DinB fingers domain. These residues are highly conserved in DinB, but not in other translesion DNA polymerases. Using a novel FRET-based assay, we found that DinB variants with mutations in these conserved residues are less effective at stabilizing RecA-mediated strand exchange than native DinB. Furthermore, these variants are specifically deficient in strand displacement in the absence of RecA filament. We propose that the amino acid patch of highly conserved residues in DinB-like proteins provides a mechanistic explanation for DinB’s function in strand exchange and improves our understanding of recombination by providing evidence that RecA plays a role in facilitating DinB’s activity during strand exchange.

Bacterial genomes accumulate lesions due to a variety of endogenous and exogenous DNA damaging agents, which result in replication fork stalling and ultimately mutagenesis (1, 2). Genomic stability is critical for cell survival and bacteria use high fidelity repair pathways, such as homologous recombination (3, 4), to ensure this stability.

Homologous recombination’s main function is to repair double strand breaks (DSBs) resulting from DNA damage and replication fork stalling (homologous recombination reviewed in (4)). In this pathway, the RecBCD complex binds both ends of a DSB and degrades the double-stranded DNA (dsDNA) until it encounters a Chi site (4–6), a hotspot for homologous recombination that consists of an eight base pair

¹ DinB, DNA polymerase IV; DSB, double strand break; dsDNA, double-stranded DNA; D-loop, displacement loop; ssDNA, single-stranded DNA, C66, DinB cysteine 66; R38, DinB arginine 38; V53, valine 53; M57, methionine 57; P67, proline 67; A62, alanine 62; DinB(C66A), DinB variant with single amino acid mutation cysteine 66 to alanine; DinB(R38A), DinB variant with single amino acid mutation arginine 38 to alanine; ΔF, change in fluorescence; nt, nucleotides; MSA, multiple sequence alignment; ΔL, distance between region of homology on dsDNA and fluorescent label; ΔD, distance between fluorescent label and closest end of dsDNA; N, length of homology.
sequence repeated across the genome (7, 8). Upon encountering a Chi site, the RecBCD complex changes function and begins to degrade only one strand of DNA in the 5′-to-3′ direction, creating a long 3′ overhang (4, 5, 9), which is in turn coated by RecA giving rise to the RecA nucleoprotein filament (10, 11). This filament performs the search for homologous dsDNA (4).

The next step is formation of a displacement loop (D-loop), in which single-stranded DNA (ssDNA) in the RecA nucleoprotein filament pairs with homologous dsDNA. In the D-loop the ssDNA displaces a strand of the dsDNA, known as the displaced strand. The stability of the D-loop is dependent on homology, which provides the system with the potential for reversibility in the case of a low homology match (12–15). When a D-loop with enough homology is formed, a DNA polymerase binds and extends the D-loop using the ssDNA as primer and the complementary strand as template (4). The strand exchange products remain reversible until there is no longer ssDNA available (12–14, 16–18), but the probability of reversal is potentially lowered with each nucleotide added.

High fidelity pathways are overwhelmed when DNA damage is severe and replicative DNA polymerases encounter lesions in the template DNA, which causes potentially lethal replication fork stalling (1, 19). Under these conditions cells resort to low fidelity DNA damage tolerance pathways (e.g. translesion synthesis) to survive. Translesion synthesis DNA polymerases synthesize across from lesions on the template strand that would otherwise stall replicative DNA polymerases (e.g. DNA polymerase III) and cause cell death. DNA polymerase IV (DinB), a highly conserved translesion DNA polymerase, bypasses N2-deoxyguanosine adducts (20–23) and alkylolation lesions (20, 24–26). DinB’s active site, which is wider than that of higher fidelity DNA polymerases, allows it to accommodate and bypass these lesions (27, 28). Conversely, DinB’s active site does not allow for the same geometric error checking that is observed in high fidelity DNA polymerases (27, 28), which causes DinB to misincorporate nucleotides and create -1 frameshift mutations (29, 30).

Stressful environmental conditions can trigger a switch from high-fidelity DSB repair, which uses DNA polymerase III to extend the D-loop, to error-prone DSB repair, which uses DinB (31–34). Interestingly, the two other highly conserved translesion DNA polymerases expressed in E. coli, DNA polymerases II and V, do not participate in RecA-mediated strand exchange during error-prone double strand break repair (35). In fact, these polymerases have been found to inhibit D-loop extension in vivo and in vitro (33, 36–39). While mechanistic details of DinB’s role in RecA-mediated strand exchange are unknown, previous work suggests that extension of the initiating strand by DinB stabilizes D-loops and that DinB’s unusually high intracellular concentration (2500 nM under DNA damaging conditions (29)) helps it to access the site of strand exchange (35). This is consistent with new work indicating that most DinB molecules carry out DNA synthesis outside replisomes (35, 40).

To gain insights into the mechanism of DinB synthesis during strand exchange, here we identify a patch of DinB residues located in the DinB fingers domain that are predominantly conserved in DinB-like proteins. We performed structural modelling, which predicts that these residues are likely positioned between the dsDNA template and displaced strands following D-loop formation, possibly contributing to or stabilizing displacement of the displaced strand.

Among these conserved residues are DinB cysteine 66 (C66) and arginine 38 (R38). We have previously shown that C66 is important for DinB’s interaction with RecA (25, 26).

Structural modelling predicts that C66 is located near the displaced strand, while R38 is near the template strand during strand exchange. We report here that both C66 and R38 are important for DinB to perform strand displacement. The evidence shown in this report provides both mechanistic insights into the role of DinB in RecA-mediated strand exchange and a possible explanation for DinB’s function as the only known translesion DNA polymerase to promote this process.

RESULTS

Molecular modelling of DinB in RecA-mediated strand exchange indicates the DinB fingers domain separates dsDNA

To further the understanding of how DinB facilitates strand exchange during DSB repair, we performed molecular modelling of DinB interacting with a RecA nucleoprotein filament and dsDNA (Figure 1A). In this model, the RecA-DNA portion represents the D-loop formation stage of homologous recombination (i.e. when
the 3’ end of the incoming nucleoprotein filament anneals to the template strand, thereby separating the dsDNA). Our model of DinB interaction at this stage reveals how DinB likely interacts with the RecA filament and dsDNA before DinB extends the D-loop (Figure 1A). The model suggests that upon DinB binding, the template and displaced strands of the heteroduplex are separated by DinB’s fingers domain (highlighted in yellow, Figure 1B). Here, we investigate whether this separation of the dsDNA is part of how DinB facilitates strand displacement through DNA synthesis during strand exchange.

Several residues predominantly conserved in DinB are predicted to be near the template or displaced strands during strand exchange.

Since DinB is the only known translesion DNA polymerase that can extend strand exchange products, we used multiple sequence alignments to investigate the conservation of the DinB fingers domain. Several residues in this area of DinB are highly conserved in DinB-like proteins (magenta and blue residues in Figure 2A) but are not conserved in translesion DNA polymerases V (Figure 2B) or II (Figure 2C). Our structural modelling predicts several of these conserved residues to interact with either the displaced (valine 53, V53; methionine 57, M57; cysteine 66, C66; proline 67; P67) or the template strand (arginine 38, R38; alanine 62, A62) during RecA-mediated strand exchange (magenta and blue residues in Figure 3). The notion that these residues, which appear to separate the dsDNA during strand exchange, are only found in DinB-like proteins suggests an explanation for why DinB is the only known translesion DNA polymerase that extends strand exchange products.

Notably, the conserved C66 in DinB is predicted to be adjacent to the displaced strand during strand exchange (blue residue in Figures 2A and 3). We have previously reported that this residue is important for DinB interaction with RecA during translesion DNA synthesis (41). The DinB(C66A) variant maintains comparable DNA synthesis activity to the native enzyme (Supplemental Figure 1; (42)); however, it demonstrates increased interaction with RecA and decreased fidelity on damaged DNA templates (25, 26). In addition, C66 is hypothesized to be important for the structure of the DinB fingers domain (42). Mutating this residue in silico to a smaller amino acid appears to eliminate an interaction between C66 and phenylalanine 51 in the fingers domain (Supplemental Figure 2). This may cause flexibility previously observed in the DinB(C66A) variant, which does not appear to significantly affect the DNA synthesis activity of the enzyme (Supplemental Figure 1; (42)).

Because of C66’s previously identified importance to the DinB-RecA interaction (25), its predicted proximity to the displaced strand during strand exchange (Figure 3), and its potential importance for the structural stability of the fingers domain (Supplemental Figure 2; (42)), we investigated C66’s role during RecA-mediated strand exchange.

Residue C66 is important for strand exchange activity

To measure strand exchange, a RecA-ssDNA filament was mixed with labelled dsDNA (Figure 4A). The number of homologous bases between the 3’ end of the ssDNA filament and the dsDNA was varied from 20-75 bp (Figure 4A). The template strand of dsDNA was labelled with rhodamine (yellow circle, Figure 4A) at a location 5 bp beyond the end of the region of homology. When annealed to the displaced strand, this rhodamine quenched a fluorescein label located nearby on the displaced strand of the dsDNA (white star, Figure 4A). As the RecA-ssDNA filament invaded the dsDNA, DinB used the ssDNA as a primer and the complementary strand in the dsDNA as a template for extension. As the template and displaced strands were separated by the polymerase, fluorescence quenching became less efficient and the fluorescence signal increased. An increase in fluorescence above the baseline indicated that (1) the initial strand exchange product was stable enough to allow DinB time to bind and (2) that DinB could synthesize enough nucleotides to disrupt the dsDNA at the location of the fluorophores. In addition, as DinB synthesized beyond five nucleotides, each nucleotide insertion may have acted to stabilize the strand exchange product, as the region of homology between the ssDNA and the template strand increased.

Disruption of dsDNA was dependent on the presence of RecA as there was little observable change in fluorescence (from time zero; ΔF) when RecA was absent (Figure 4B, DinB only). We observed a non-zero baseline of fluorescence with only RecA, suggesting that
RecA altered the dsDNA at least 5 bp beyond the 3' end of the filament (Figure 4B, RecA only N=75). This may be caused by an alteration in the topology of the DNA that occurs as RecA forms the D-loop. As expected, addition of DinB and no dNTPs did not significantly alter the RecA baseline fluorescence due to the lack of DNA synthesis (Figure 4B, RecA, DinB. No dNTPs). In addition, a maximum ΔF for this assay was measured using a ssDNA filament that contained a full 90 bp of homology to the dsDNA (Figure 4B, RecA only N=90).

RecA, dNTPs, and DinB synthesis disrupted the dsDNA, which in turn relieved quenching and increased fluorescence in experiments with different lengths of homology (see increasing fluorescence in Figure 4C). The increase in fluorescence due to the presence of DinB was dependent on the homology between the ssDNA filament and the dsDNA. We note that 75 bp of homology was required to observe a fluorescence increase due to DinB synthesis (compare Figure 4C to RecA, DinB, no dNTPs control in Figure 4B). This indicates that under the conditions of the experiment 75 bp of homology was required for the RecA-mediated strand exchange product to be stable enough for DinB to alleviate quenching at the fluorophores in the dsDNA. Lower homology (<75 bp) does not allow for DinB binding and synthesis.

Although DinB(C66A) primer extension was comparable to the native enzyme’s (Supplemental Figure 1; (25, 26)), it was not as proficient in the strand exchange assays (Figure 4D). While disruption of dsDNA was dependent on the length of homology between the ssDNA RecA filament and the dsDNA, synthesis by DinB(C66A) with 75 bp of homology disrupted the dsDNA far less than that of DinB (compare blue lines in Figures 4C and 4D). This is indicative of lower DNA synthesis during strand exchange by DinB(C66A) than the native enzyme.

In these experiments, the initial rate is a measure of the rate at which the enzyme can disrupt dsDNA and cause a change in fluorescence. The maximum ΔF is indicative of the stability of the strand exchange product at the location of the fluorescent labels, as it is a point of equilibrium between separated and annealed dsDNA.

A direct comparison of the reactions using 75 bp of homology (Figure 5A) is highlighted in Figure 5B. In this assay DinB(C66A) had a slightly (~20%) lower initial rate (ΔF/s) than DinB (Figure 5B; p < 0.0001; enlargement of Figure 5B from 0-200 s is provided in Supplemental Figure 3), indicating that the rate at which the dsDNA was initially separated was approximately only 20% slower than for DinB. This difference is possibly due in part to minor differences in the enzymes’ rate of primer extension (Supplemental Figure 1). However, the maximum fluorescence reached by DinB(C66A) was ~50% lower than that reached by DinB (Figure 5B; p < 0.01). This indicates that the stability of the resulting strand exchange products was poorer due to less DNA synthesis and likely reversed more often than those created by DinB. These data suggest that while DinB(C66A) initially separated the dsDNA 20% more slowly, its ability for DNA synthesis at some point beyond the fluorophores either slowed down further or stopped prematurely to result in a less stable strand exchange product. The observed slowed down cannot be accounted for simply by the slight differences in the enzyme’s primer extension activity.

**DinB(C66A) can insert a single nucleotide during strand exchange as well as DinB.**

To further investigate the mechanisms underlying DinB’s strand exchange activity, we used dsDNA containing a fluorescent label located only a single base pair outside of the homologous region (Figure 6A). In this experiment, DinB must insert only a single nucleotide to separate the rhodamine (on the template strand) from the fluorescein (on the displaced strand) and increase fluorescence. When only a single nucleotide insertion was required (Figure 6B), DinB(C66A) performed similarly to native DinB (compare Figure 5B and 6B). The initial rate of the DinB curve was not significantly different from that of DinB(C66A) (enlargement of Figure 6B from 0-50 s is provided in Supplemental Figure 3). Notably, the DinB curve did reach a significantly higher maximum ΔF (p < 0.01). This again suggests that while there is little to no difference in the initial rates of dsDNA separation, the final strand exchange products made by DinB(C66A) are less stable (i.e. they reverse more often) and therefore likely had lower final homology.

While only a single nucleotide insertion is required to observe fluorescence in this assay, the presence of all dNTPs allows for more than one insertion. Each nucleotide insertion, in turn, may increase the stability of the strand exchange product, thereby preventing a decrease in fluorescence that is caused by the displaced...
strand reannealing the template. Since initial rates of the curves indicate that DinB and DinB(C66A) insert the first nucleotide at a similar rate, we hypothesized that the difference in maximum ΔF in Figure 6B was due to difference in DNA synthesis after the first insertion.

To observe only a single nucleotide insertion, the experiment was repeated using only the dNTP required for the first insertion dATP. Interestingly, we find that DinB(C66A) performed the first nucleotide insertion at a slightly higher rate (p < 0.05; Figure 6C, enlargement of Figure 6C from 0-100 s is provided in Supplemental Figure 3) than DinB. This is consistent with previous work, which indicates that DinB(C66A) inserts dATP with greater efficiency than the native enzyme (41). In addition, DinB(C66A) was previously discovered to bind more efficiently to RecA (42), which may facilitate the DinB(C66A) binding to the intricate DNA substrate in this assay. These data suggest that DinB(C66A) can insert the first nucleotide during strand exchange with at least the same efficiency as DinB.

In addition, here we eliminate the potential for differences in DNA synthesis beyond the fluorophores and find no significant difference in the maximum ΔF reached by the two enzymes (Figure 6C). This supports our hypothesis that the difference in maximum ΔF in previous assays was due to a difference in DNA synthesis beyond the fluorophores.

To rule out previously observed differences between DinB and DinB(C66A) fidelity (26) as the cause of differences in activity, we tested the fidelity of both proteins using each single dNTP under the experimental conditions shown in Figure 6A. In these experiments, the first nucleotide insertion by DinB should be a dATP across from a dTTP on the template strand. We find that DinB inserted dATP well; the increase in fluorescence for the dATP insertion was comparable to when all dNTPs are present (Supplemental Figure 4). When an incorrect dNTP for the insertion was provided (Supplemental Figure 4), the fluorescence was comparable to the RecA only (no DinB) fluorescence (Supplemental Figure 4) and to the RecA only (no DinB, no dNTPs) fluorescence (Supplemental Figure 4). These experiments were also performed in conditions requiring five nucleotide insertions (Supplemental Figure 5) and comparable results were observed, indicating that differences in fidelity between the enzymes is not detectable in the fluorescence readings of these assays.

These data show that the DinB(C66A) variant can perform the first nucleotide insertion during strand exchange at least as efficiently as the native enzyme.

**DinB(C66A) variant is deficient in strand displacement**

To understand the difference between DinB(C66A)’s high efficiency in inserting a single nucleotide and low efficiency in separating dsDNA 5 bp beyond the ssDNA filament, we consulted our model of DinB interactions during strand exchange (Figure 1). Our modelling indicates that upon DinB binding, the template and displaced strands of the heteroduplex are separated by DinB’s fingers domain (Figures 1B and 3). In this manner, 1-2 nucleotides (nt) of the template strand that lie directly beyond the 3’ end of the invading RecA nucleoprotein filament are “free” (Figure 3) and prevented from annealing to the displaced strand (orange strand in Figures 1B and 3). This information suggests a possible explanation for the DinB(C66A)’s observed activities. We hypothesized that DinB(C66A) can efficiently synthesize using a free template (24, 25; Supplemental Figure 1); however, it would not be able to separate the displaced strand during strand exchange. To test this, we performed strand displacement experiments in the absence of RecA. In these experiments, DinB must displace the strand that is annealed to the template, without the help of RecA, before it can synthesize DNA.

To our knowledge, DinB’s innate ability to perform DNA synthesis with strand displacement in the absence of RecA has not been previously investigated. To do this experiment, a ssDNA template (90 nt bottom black line in Figure 7A) was annealed to a ssDNA primer (29 nt grey line in Figure 7A) and a 75 nt ssDNA that mimics a displaced strand (75 nt top black line in Figure 7A). Only 61 nt of the 75 nt ssDNA were complementary to the template strand. The other non-complementary 14 nt formed a short flap of un-annealed ssDNA on the 5’ end of the 75 nt ssDNA (Figure 7A). This strand was labelled with fluorescein at the nucleotide directly following the 3’ end of the un-annealed flap (Figure 7A). As primer extension occurs, the displaced strand is separated from the template strand; therefore, fluorescence of the fluorescein
label changes as the displaced strand moves away from the template strand.

When the fluorescein is located directly beside the end of the primer (Figure 7A), DinB must insert only a single nucleotide to separate the fluorescein-containing nucleotide on the displaced strand. In this case, DinB showed a short lag before efficiently separating the displaced strand (black line in Figure 7B, an enlargement is provided in Figure 7C to highlight the lag). During the first 50 s, there was no significant fluorescence increase. This lag likely coincides with DinB binding to the DNA before DNA synthesis. Notably, this lag was absent in all RecA-dependent experiments, possibly because the RecA-DinB interaction facilitates DinB binding to the intricate DNA template.

DinB(C66A) demonstrated a similar lag as DinB (Figure 7B, enlargement provided in Figure 7C; the rate from 0-50 s is not significantly different from zero). While DinB(C66A) has shown similar activity to native DinB in primer extension assays (25, 26), DinB(C66A) was significantly less proficient at DNA synthesis during strand displacement. The rate from 100-200 s was about 75% lower than the same interval in DinB’s reaction (p < 0.0001) and the DinB(C66A) reaction reached a maximum ΔF that is about 65% lower than DinB (p < 0.05).

Together these data indicate that DinB can synthesize from a primer in templates with a topology that mimics strand displacement in recombination intermediates. Remarkably, this ability is independent of RecA. In addition, residue C66, which is highly conserved in only DinB-like proteins, is critical to DinB’s ability to perform strand displacement.

DinB(C66A)-containing strain induces significantly less mutagenesis during DSB repair

DinB activity during error-prone DSB repair has been shown to cause mutagenesis (31–33, 35). Error-prone DSB repair-induced mutagenesis is significantly reduced in a ΔdinB strain (31). Ponder et al. (31) have previously described a system with which to assess this DinB dependent mutagenesis. A chromosomal reporter was constructed that encodes an arabinose-inducible I-SceI endonuclease (31), which creates a DSB at an 18 bp I-SceI recognition site, located ~11.4 kb downstream from a lac gene containing the LacI333 allele. Cells with this allele are Lac− (i.e. unable to use lactose as a sole carbon source) because of a +1 frameshift in the lac gene (31). The reversion from Lac− to Lac+ is measured by counting Lac+ colonies on lactose containing minimal medium (31). Ponder et al. showed that deletion of the dinB gene significantly reduced the number of Lac− reversion (31). Here we confirm these results and find that the test E. coli strain with a chromosomal dinB(C66A) allele at the dinB locus produces significantly fewer Lac+ revertants than the dinB+ strain (Figure 7D; 5-10 fold decrease, p < 0.05 at all time points). These data support our model that DinB(C66A) is not as proficient as DinB during error-prone DSB repair in vivo. This experiment also provides a proof of principle that the obtained in vitro data correlates well with the in vivo behaviour of the enzyme.

Template binding DinB residue R38 is also important for strand displacement.

To further our understanding of the role of the fingers domain in DinB’s synthesis strand exchange, we investigated another of the highly conserved residues that we identified by modelling. Like C66, R38 is predominantly conserved in DinB-like proteins. While our structural modelling predicts C66 to be located near the displaced strand in strand exchange, R38 is predicted to be located near the template strand. Arginine’s positive charge makes it an excellent candidate for interaction with the negatively charged DNA strand. To test the importance of this residue to DinB’s strand exchange activity, we constructed a variant in which only this residue was mutated to an uncharged alanine (DinB(R38A)). We found that the DNA synthesis activity using an undamaged DNA template is comparable to that of the native enzyme (about 20% lower than native DinB activity, Supplemental Figure 1). Like DinB(C66A), the DinB(R38A) derivative stabilized strand exchange products but did so with reduced efficiency compared to native DinB (compare Figure 8A to Figure 4C).

When only one nucleotide insertion was required to separate fluorophores and all dNTPs were provided (as in Figure 6B), DinB(R38A) had an 18% lower initial rate and a 40% lower maximum ΔF than native DinB (Figure 8B; p < 0.0001, p < 0.01).

When only the dATP was provided, difference in initial rate between the two enzymes remained similar (about 25% Figure 8C, p < 0.0001), while the maximum ΔFs were not significantly different (Figure 8C). These data
suggest that R38 is important for DNA synthesis during strand exchange. Again, we hypothesized that while DinB(R38A) can insert nucleotides across from a free template, it cannot efficiently separate a displaced strand to insert nucleotides.

Notably, we find that DinB(R38A) was fully deficient in strand displacement activity in the absence of RecA (Figure 8B; experimental setup shown in Figure 7A). Since our modelling (Figure 3) and the crystal structure of DinB during primer extension (43) indicate that this residue is located near the template strand during strand exchange and primer extension, respectively, it is likely that DinB(R38) aids strand displacement by stabilizing the template strand during DNA synthesis.

In addition, these data indicate an additional active role for RecA in strand exchange beyond simply forming a D-loop, since DinB(R38A) can perform some DNA synthesis during RecA-dependent strand exchange, but is not able to perform any detectable DNA synthesis during RecA-independent strand displacement.

**DISCUSSION**

Stressful environmental conditions can trigger a switch from high-fidelity DSB repair, which uses DNA polymerase III to extend the D-loop, to error-prone DSB repair which uses DinB for this function (31–34). DinB is the only of three *E. coli* translesion DNA polymerases known to promote RecA-mediated DSB repair (35). In fact, the other *E. coli* translesion DNA polymerases II and V actually inhibit this process (33, 36–39). In addition, DinB significantly improves the efficiency of recombination events during transduction, a horizontal gene transfer process, while translesion DNA polymerase V appears to inhibit these events (42).

Our model of DinB’s interaction with DNA during RecA-mediated strand exchange predicts that the DinB fingers domain lies directly between the template and displaced strands during strand exchange. Interestingly, several residues in this domain are highly conserved in DinB, but not in the translesion DNA polymerases that inhibit strand exchange (Figure 2). Notably, our molecular modelling indicates that several of the conserved residues in the fingers domain of DinB are located near the template strand (R38 and A62, Figure 3) or the displaced strand (V53, M57, C66, and P67, Figure 3) during strand exchange. The conservation and location of these residues suggests a structural explanation for DinB to be the only *E. coli* translesion DNA polymerase with a known role in RecA-mediated strand exchange.

Our group previously showed the C66 residue is highly conserved among DinB-like proteins (42). We also showed that it is important to RecA-dependent increase in fidelity during translesion synthesis (41, 42), and in DinB’s ability to promote recombination during transduction. Furthermore, we show here that C66 is central to DinB’s ability to perform strand displacement during RecA-mediated strand exchange. Notably, we have previously predicted that DinB was likely to interact with the RecA nucleoprotein filament (44), and not just with RecA alone, and here we showed that to be the case.

We have found that the DinB(C66A) variant is more flexible in the fingers domain than the native enzyme; however, it does not appear to be degraded *in vivo* and the catalytic core of the enzyme is not significantly affected (25). Analysis of DinB’s crystal structure (43) reveals that C66 appears to interact with phenylalanine 51 (F51; Supplemental Figure 2). The orientation of the atoms of C66 in relation to the benzene ring of F51 suggests that these two residues may form an aromatic-thiol π-type hydrogen bond (45), which would be disrupted in the DinB(C66A) variant. The loss of this bonding may explain the structural flexibility that was previously observed (25). It is likely that C66 is important to DinB’s ability to perform strand displacement, because of its stabilization of the structure of the fingers domain and not just through direct interaction between C66 and dsDNA. The importance of C66 in the structure of the fingers domain may also explain its importance to the many DinB functions outlined above.

The crystal structures of DinB bound to a primer and an undamaged (43) or lesion-containing (46) template indicate that R38 interacts with the template strand. Our structural model predicts R38 to interact with the template strand during strand exchange as well (Figure 3). In addition, a previous study characterizing the DinB(R38A) variant determined that R38 is responsible for suppressing misincorporations during bypass of N2-deoxyguanosine adducts (47). Given the complete lack of DinB(R38A) strand displacement activity *in vitro*, it is probable that this highly conserved residue is involved in template DNA binding during strand displacement.
We observed here that native DinB experiences a significant lag before starting DNA synthesis during strand displacement in the absence of RecA (Figure 7C). Presumably this lag is caused by a lower affinity of DinB for this intricate DNA substrate. Interestingly this lag is absent when RecA is available to form the D-loop before synthesis (Figure 6B). Additionally, the DinB(R38A) mutant can perform RecA-dependent strand exchange, while it is completely devoid of strand displacement function in the absence of RecA. These data suggest that RecA facilitates DinB’s activity during strand exchange. One possible mechanism for this would be that RecA recruits DinB to the site of strand exchange by the protein-protein interaction that we have previously observed (41, 42, 44). Here we observe that RecA affects the annealing of dsDNA at a location that is 5 bp beyond where 3’ end of nucleoprotein filament binds (Figure 4B). This suggests that RecA alters the topology of the dsDNA once the D-loop has formed, which may facilitate DinB’s binding to this intricate DNA substrate and the progression of strand displacement required for synthesis.

Our observations provide mechanistic insights that support DinB’s hypothesized role as the DNA polymerase that initially performs primer extension at the D-loop during certain replication fork stalling events (35). The C66, R38, and likely the surrounding amino acid patch that are predominantly conserved (Figure 2) in DinB and DinB-like proteins are important for strand displacement (Figure 7). Taken together, the data outlined here suggest a similar role for this conserved amino acid patch in strand displacement by separating the template and displaced strands of dsDNA.

EXPERIMENTAL PROCEDURES

Protein purification

DinB was purified from both the TMCAT strain (BL21-AI ΔdinB, ΔumuDC, ΔrecA; (48)) by ion exchange chromatography and hydrophobic interaction chromatography as previously described (25, 48–50). DinB(C66A) was purified from the TMCΔD strain (BL21-AI ΔdinB, ΔumuDC; (26)) using an identical methodology.

Strand exchange assay

RecA nucleoprotein filament was formed by adding ssDNA (0.06 μM; Tables 1 and 2), RecA (2 μM; New England Biolabs, Ipswich, MA), dATP, (1 mM), pyruvate kinase (10 U/mL), phosphoenolpyruvate (3 mM), and single-stranded binding protein (SSB; 0.2 μM, Epicentre, Maddison, WI) in RecA buffer (70 mM Tris-HCl, 10 mM MgCl2, and 5 mM dithiothreitol, pH 7.6) at 37°C for 10 minutes.

The dsDNA constructs (Tables 1 and 2) were prepared by annealing the complementary oligonucleotides at temperatures from 90°C to 40°C with 1°C steps equilibrated for 1 minute in a thermocycler.

For the strand exchange reactions 0.06 μM 98 nt ssDNA/RecA filament was mixed with 0.06 μM rhodamine-fluorescein labelled dsDNA, and E. coli DinB. The mixture was rapidly transferred to a quartz cuvette. For DinB measurements, the RecA buffer contained 0.1 mg/mL BSA, 2 mM dATP, and 1.0 mM dNTPs (or 1 mM of each required dNTP). The final polymerase concentration was 100 nM in all experiments.

FRET experiments were performed with a Fluoromax 4C spectrofluorometer (Horiba, Edison, NJ) by following the emission of the fluorescein label at 518 nm every one second for 30 minutes and 493-nm excitation. The integration was 0.5 s and the band width 2 nm. Samples were kept at 37°C.

Molecular Modelling

The DinB-RecA-DNA complex was assembled as follows. The RecA-DNA part represents the homologous recombination stage where strand exchange has taken place up to the 3’ extremity of the incoming strand. The heteroduplex product of strand exchange is bound to the filament DNA-binding site I and the displaced strand is in the filament binding site II. The DNA in site I was directly taken from the crystal structure with PDB code 3CMW (51), which has been extended to 3 filament turns using the PTools library (52). The structure of the displaced strand was taken from former modelling work where we simulated the early stage of strand exchange (53). The geometry of the junction between the heteroduplex 3’ extremity and the double-stranded B-form DNA tail (D-loop junction), formed by the complementary strand and the displaced strand, was also taken from that former work, such as it resulted from spontaneous strand exchange. The B-form tail interacts with the filament groove, notably with the C-terminal domain of the RecA monomer at the 3’ extremity of the filament. The three-turn filament with three
DNA strands included was stable during 100 ns molecular dynamics simulation in solvated environment at 300 K, performed with NAMD 2.10 (54) and the CHARMM 27 force field (55). The geometry of DinB in complex with the RecA filament was obtained by superposing the terminal four base pairs of the DNA in the crystal structure of the DinB/DNA complex with PDB code 4IRC (43) on the four terminal heteroduplex base pairs. Despite the heteroduplex being strongly unwound and stretched with respect to B-form DNA, the superposition was made possible by the fact that the heteroduplex is structured as a succession of base pairs triplets in the B-form, separated by intercalation sites which concentrate the stretching/unwinding deformations. Steric overlaps that resulted from the brute superposition of DinB-DNA on the heteroduplex, notably between DinB and the displaced strand, the DinB thumb and a region of the last RecA monomer close to loop L1, the little finger and the L2 loops of the last two RecA monomers, were removed using interactive and flexible simulations with the BioSpring device and associated software (56). These simulations did not use any knowledge-based restraint. When adjusting the complementary and outgoing strand regions situated in 3’ of the D-loop junction, we were careful to minimally displace the strands from their initial relaxed position in the RecA filament. Adaptation of the complementary strand to the presence of DinB necessitated its backbone displacement over a 6 base region, thus locally following the direction taken by the template strand in the DinB complex; this displacement was compatible with conserving most of the Watson Crick interactions with the outgoing strand, which itself varied little from its geometry in the DinB-free RecA filament.

**RecA-independent strand displacement synthesis**

Experiments were performed in RecA buffer at 37°C. A solution containing DNA template (200 nM), dNTPs (1 mM), BSA (0.1 mg/mL), and *E. coli* DinB or DinB(C66A) (100 nM) was transferred to a quartz cuvette. Measurements of fluorescein emission at 37°C were performed at 518 nm (excitation at 493 nm). Oligonucleotides used in these experiments are described in Table 3.

**Multiple Sequence Alignments**

Multiple sequence alignments (MSAs) of DinB, DNA polymerase V, and DNA polymerase II sequences were performed using CLC Bio (Qiagen, Redwood City, CA). DinB and DNA polymerase V sequences were obtained from NCBI and hand curated using previously described criteria (25). The DinB MSA utilized 472 sequences from over 100 bacterial species; the DNA polymerase V MSA utilized 2694 sequences from over 600 bacterial species. The DNA polymerase II sequences were obtained from NCBI and hand curated to the following criteria: were annotated as a DNA Polymerase II and were at least 600 amino acids long and shorter than 900 amino acids long. The DNA polymerase II MSA utilized 124 sequences from over 100 bacterial species.

**In vivo DSB-induced mutagenesis assay**

The DSB-induced mutagenesis assay was performed as previously described (31). The dinB(C66A) allele was mobilized by P1 transduction (57) from MG1655 dinB(C66A) (25) into the assay strain (31). Strains to be tested were streaked from frozen stock on M9 medium supplemented with vitamin B1 with glucose as sole carbon source and grown for two days at 37°C. Twelve single colonies per strain were inoculated into M9 liquid medium supplemented with vitamin B1 and grown for 48 hrs at 37°C until saturation. Cultures were then deposited on the surface of M9 medium plates supplemented as above but with Lactose as sole carbon source to measure Lac⁺ revertant colonies. Viable cell measurements were performed on the same kind of M9 medium plates with glucose as sole carbon source. Revertant colony counts were performed at 3, 4, and 5 days after plating.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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**Table 1.** Oligonucleotides used in RecA-dependent strand exchange assays in Figure 4 and 5. F*, fluorescein-labelled nucleotide; Rho*, rhodamine-labelled nucleotide. All oligonucleotides purchased from Integrated DNA Technologies (Coralville, IA)
### Oligonucleotides for RecA-dependent strand exchange (Figures 6)

| Sequence | Role | Homology with 90 bp dsDNA (bp) | Distance between region of homology and fluorophore (nt) |
|----------|------|-------------------------------|--------------------------------------------------------|
| ACTGCTTCATTCCCTTATTACCTGCACCTCCACGC | ssDNA filament | 79 | - |
| CACTGAGGTATGCCGCATTGCACCTTGTCCCTGG | dsDNA displaced strand | - | 1 |
| CAGTGGTCGTCTCTTTTCATATACCGG | dsDNA template strand | - | 1 |
| TACCTGCACTCCACGCCACTGAGGTATGCCGATT | | | |
| GCACTTTTGTCCCTGGGAGTGGTGTCGTCTCTTTCAT | | | |
| ATACCAGGGAG-T(F* )-GATTTCCG | | | |
| 5´CGGAAATCAC-T(Rho*)-CCCGGTATATGAA | | | |
| AGAGACGACACCATGCGAGGACGAAATGCAATGC | | | |
| GGCATAACTGATGCGGATGAGTGCAGGTA | | | |

**Table 2.** Oligonucleotides used in RecA-dependent strand exchange assays in Figure 6. F*, fluorescein-labelled nucleotide; Rho*, rhodamine-labelled nucleotide. All oligonucleotides purchased from Integrated DNA Technologies (Coralville, IA).
### Oligonucleotides for RecA-independent strand displacement (Figure 7)

| Sequence                                      | Role                  |
|-----------------------------------------------|-----------------------|
| TACCTGCACCTCCACGGTGACTGTACGAA                | Primer                |
| CCACTGAGGTATG-<sup>F</sup>* -GCATTGCAC       | Displaced strand with flap |
| TTTGCTCCGAGGATGGTCTCTCTTC                    |                       |
| ATATACCGGAGTGTATTTCG                        |                       |
| CGGAAATCCTCCGAGGATTATGAAAGAGACGACC          | Template strand       |
| ACTGCCAGGGGCAAGTGCAATGCTACG                 |                       |
| TACACCGTGAGTGCAGTA                         |                       |

**Table 3.** Oligonucleotides used in RecA-independent strand displacement assays in Figure 7. F*, fluorescein-labelled nucleotide. All oligonucleotides purchased from Integrated DNA Technologies (Coralville, IA)
Figure 1. Molecular modelling of DinB in RecA-mediated strand exchange indicates the DinB fingers domain separates dsDNA. (A) This model of RecA-DNA-DinB interaction represents the homologous recombination stage where strand exchange has taken place up to the 3’ extremity of the incoming strand. Interactions were modelled using structures and models as follows. The ssDNA (grey, largely obscured by RecA), template strand of dsDNA (black), displaced strand of dsDNA (orange), and RecA (cyan) were modelled using the PDB structure 3CMW (51) that had been extended by three turns (52) in a previous model (53). DinB (green and yellow) was modelled using the PDB structure 4IRC (43). The RecA nucleoprotein filament and DinB are shown interacting with the RecA molecule that lies at the 3’ end of the nucleoprotein filament. DinB and the RecA nucleoprotein filament were surface rendered with PyMol (58). The fingers domain of DinB is highlighted in yellow. (B) DinB and DNA are shown without RecA to permit visualization of DinB’s interaction with three DNA strands during strand exchange. Structures have been turned approximately 180° about the horizontal axis with respect to (A). DinB’s fingers domains (yellow) appears to separate the template (black) and displaced (orange) strands of the dsDNA.
Figure 2. Several residues of the DinB fingers domain are highly conserved only in DinB-like proteins. Multiple sequence alignments show that an amino acid patch, depicted here in magenta and blue (A) located in the fingers domain of DinB are highly conserved in DinB-like proteins, but not in DNA polymerase V (B) or in DNA polymerase II (C) homologs. Percent conservation values for A-C are reported in Supplemental Table 1.
Figure 3. Modelling suggests that several conserved DinB residues are located near the template (black) and displaced (orange) strands during strand exchange. Depiction of DinB from surface rendered model from Figure 1 shows that cysteine 66 (blue) and other predominantly conserved residues only in DinB-like proteins (magenta) are located near the template (R38, A62) and displaced (V53, M57, C66, and P67) strands of dsDNA during strand exchange. Enlargement of boxed area provided to better visualize the positions of the highlighted residues. Residues in green, magenta, and blue color correspond to residues of the same color in Figure 2.
Figure 4. Highly conserved fingers domain residue is important for DinB’s activity during strand exchange. (A) Schematic shows experimental setup for RecA-dependent strand exchange experiments. Fluorescently labelled dsDNA (left) is mixed with each of a set of ssDNA RecA filaments (right). Each in the set of filaments contains a different length of homology (N) to the labelled dsDNA (signified by grey, green, magenta, and blue lines). In the dsDNA, the template strand is labelled with rhodamine (yellow circle) and the displaced strand is labelled with fluorescein (white star). These fluorophores are located 5 bp away from area of homology between dsDNA and ssDNA RecA filament (ΔL = 5 bp). The proximity of the rhodamine quenches fluorescein fluorescence until template and displaced strands are separated. Fluorescence increases when the ssDNA RecA filament invades the dsDNA and DinB synthesizes DNA using the ssDNA filament as a primer. Five nucleotide insertions are needed to separate the displaced strand at the location of the fluorescent labels and relieve quenching. (B) When only RecA or only DinB is mixed with the highest homology ssDNA RecA filament (N = 75, blue filament in (A)), the fluorescent labels are not efficiently separated, indicating that the dsDNA is still annealed at the location of the labels. When both proteins are present in the absence of dCTP, dGTP, and dTTP (dATP is present for nucleoprotein filament assembly), baseline fluorescence is observed. A RecA filament with full homology to the dsDNA (N=90) is used to determine maximum possible fluorescence in the assay. (C) DinB stabilizes strand exchange in a homology-dependent manner. As homology increases between the ssDNA RecA filament and the fluorescently labelled dsDNA, DinB efficiently separates the dsDNA at the location of the fluorescent labels. This indicates that increased homology allows DinB to more efficiently stabilize strand exchange products. (D) DinB(C66A) stabilizes strand exchange products, but it does so with less efficiency than the native enzyme. N, length of homology between dsDNA and ssDNA filament; ΔL, distance between region of homology on dsDNA and fluorescent label; D_{init}, distance between fluorescent label and closest end of dsDNA; ΔF,
change in fluorescence measured in counts per second (cps) with respect to the fluorescence at 0 s. Experiments were performed in triplicate with similar results. Representative data are shown.
Figure 5. DinB stabilizes strand exchange products significantly better than DinB(C66A). (A) As in Figure 4, RecA-dependent strand exchange experiments utilize an ssDNA RecA filament and dsDNA that is fluorescently labelled on either strand to cause quenching. These fluorophores are located 5 bp away from the region of homology between the dsDNA and the ssDNA RecA filament (ΔL = 5 bp). Shown here is the ssDNA RecA filament with the highest homology, 75 nt. Experiments were performed using all four dNTPs. (B) Shown is the direct comparison between the fluorescence obtained with DinB and DinB(C66A) in experiments with 75 bp of homology between the RecA ssDNA filament and the dsDNA (directly compares blue lines from Figures 4C and D). Both DinB and DinB(C66A) stabilize strand exchange products and separate the dsDNA at the location of the labels, but DinB does this better than the variant. The DinB reaction has a significantly higher initial rate (18.59 ± 0.3395 cps) than the DinB(C66A) reaction (14.34 ± 0.2784 cps, p-value < 0.0001). The DinB reaction also reaches a significantly higher maximum ΔF (16987 ± 2519 cps) than the DinB(C66A) reaction (8893 ± 913; p-value < 0.01). N, length of homology between dsDNA and ssDNA filament; ΔL, distance between region of homology on dsDNA and fluorescent label; Dinit, distance between fluorescent label and closest end of dsDNA; ΔF, change in fluorescence measured in counts per second (cps) with respect to the fluorescence at 0 s. Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown.
Figure 6. DinB(C66A) can insert a single nucleotide during strand exchange as well as DinB. (A) RecA-dependent strand exchange experiments were performed similarly to Figure 5, except fluorophores are located 1 bp away from the region of homology between the dsDNA and the ssDNA RecA filament ($\Delta L = 1$ bp). Only a single nucleotide insertion is required to separate fluorophores and relieve quenching. (B) Similar to Figure 5, experiments used all four dNTPs. Initial rates of DinB (68.48 ± 3.769 cps) and DinB(C66A) (61.86 ± 2.306) reactions were not significantly different. The DinB reaction reaches a significantly higher maximum fluorescence (17163 ± 913 cps) than DinB(C66A) (13859 ± 580 cps; p-value < 0.01). Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown. (C) Experiments were performed as in (B), except that only dATP, the nucleotide required for a single insertion, was included. The DinB reaction has a significantly lower initial rate (56.31 ± 0.8425 cps) than the DinB(C66A) reaction (58.57 ± 0.6877 cps, p-value < 0.05). The maximum $\Delta F$s reached by DinB (9756 ± 1267 cps) and DinB(C66A) (11679 ± 180 cps) were not significantly different. Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown. N, length of homology between dsDNA and ssDNA filament; $\Delta L$, distance between region of homology on dsDNA and fluorescent label; $D_{init}$, distance between fluorescent label and closest end of dsDNA; $\Delta F$, change in fluorescence measured in counts per second (cps) with respect to the fluorescence at 0 s. Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown.
Figure 7. DinB(C66A) variant is deficient in RecA-independent strand displacement. (A) Graphic depiction of the DNA substrate used in these experiments. A 29 nt ssDNA primer (grey line) was annealed to a 90 nt template (bottom black line) as well as to a 75 nt fluorescently labelled oligonucleotide (top black line with the fluorophore represented by the star). The 75 nt oligonucleotide displaced strand is composed of a 61 nt complementary to the 90 bp template and of a 14 nt un-annealed flap located at the 5' end. The fluorescein label (depicted by the star) on the displaced strand was located on the first nucleotide of the 75 bp complementary region. If displaced and template strands are separated, fluorescence is altered. DinB must insert a single nucleotide onto the end of the primer to displace the labelled nucleotide. (B) Experiments with all dNTPs added show that DinB stabilizes strand displacement after a short lag (highlighted by the enlargement in (C)) with greater efficiency than the DinB(C66A) variant. Initial velocities for both proteins from 0-50s are not significantly different from zero. The rate of the DinB reaction from 100-200s (46.40 ± 1.188 cps) is significantly higher than the velocity of the DinB(C66A) reaction at the same time point (11.80 ± 1.140 cps; p-value < 0.0001). The DinB reaction also reaches a significantly higher maximum ΔF (28053 ± 6272 cps) than the DinB(C66A) reaction (9798 ± 1290 cps; p-value < 0.01). Maximum ΔF was measured using a measure of the fluorescence caused by the outgoing strand alone as about 37000 cps. Change in fluorescence measured in counts per second (cps) with respect to the fluorescence at 0 s. Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown in (B). Only the mean is shown in (C) for better visualization of lag. (D) An E. coli strain containing a system described by Ponder et al. (31) was used to examine mutagenesis in response to an induced DSB (left). The endonuclease I-SceI created a DSB 11.4 kb on the E. coli chromosome downstream of the lac gene. The lac gene in this strain contains a mutation preventing cell growth when the only carbon source is lactose represented here by lac<sup>mut</sup>. The Lac<sup>-</sup> to Lac<sup>+</sup> reversion is measured as the number of Lac<sup>+</sup> colonies.
observed when the test strain is grown on lactose only-containing minimal medium divided by the total number of colonies observed when the strain is grown on glucose only-containing minimal medium (right). The (C66A) mutation in DinB (red) significantly reduces the rate of Lac+ reversion in the presence of a DSB. For comparison purposes we used ΔdinB and dinB+ strains shown in grey and black respectively. Data shown is mean of 12 replicates ± standard error. * indicates a p-value < 0.05 when comparing the dinB+ and dinB(C66A) strains by two tailed t-test.
Figure 8. DinB(R38A) variant is deficient in strand displacement. (A) Reactions performed as in Figure 4. DinB(R38A) stabilizes strand exchange products, but it does so with less efficiency than the native enzyme. N, is the length of homology between dsDNA and ssDNA filament. Experiments were performed in triplicate. Mean is shown. (B) Reactions were performed as in Figure 6A with all four dNTPs. DinB has a significantly higher initial rate (68.48 ± 3.769 cps) than DinB(R38A) (55.85 ± 1.855 cps; p-value < 0.0001) in this assay. The DinB reaction reaches a significantly higher maximum fluorescence (17163 ± 913 cps) than DinB(R38A) (9949 ± 1742 cps; p-value < 0.01). Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown. (C) Experiments in which only a single insertion is required were performed as in Figure 6A using only dATP, the nucleotide required for the single insertion. DinB had a significantly higher initial rate (56.31 ± 0.8425 cps) than DinB(R38A) (40.88 ± 1.561 cps; p-value < 0.0001) in this assay. The maximum ΔFs of DinB (9756 ± 1267 cps) and DinB(R38A) (7539 ± 1470 cps) were not significantly different. Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown. ΔF, change in fluorescence measured in counts per second (cps) with respect to the fluorescence at 0 s. (D) The ability of the enzyme for strand displacement was tested as in Figure 7A with all four dNTPs. DinB(R38A) has little to no activity in this assay. Maximum ΔF was measured using a measure of the fluorescence caused by the outgoing strand alone as about 37000 cps. Experiments were performed in triplicate. Mean ± standard deviation (shaded region surrounding curves) is shown.
Residues in the fingers domain of the translesion DNA polymerase DinB enable its unique participation in error-prone double strand break repair

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