Role of *Pseudomonas aeruginosa* *dinB*-Encoded DNA Polymerase IV in Mutagenesis[∇]

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Pseudomonas aeruginosa is a human opportunistic pathogen that chronically infects the lungs of cystic fibrosis patients and is the leading cause of morbidity and mortality of people afflicted with this disease. A striking correlation between mutagenesis and the persistence of *P. aeruginosa* has been reported. In other well-studied organisms, error-prone replication by Y family DNA polymerases contributes significantly to mutagenesis. Based on an analysis of the PAO1 genome sequence, *P. aeruginosa* contains a single Y family DNA polymerase encoded by the *dinB* gene. As part of an effort to understand the mechanisms of mutagenesis in *P. aeruginosa*, we have cloned the *dinB* gene of *P. aeruginosa* and utilized a combination of genetic and biochemical approaches to characterize the activity and regulation of the *P. aeruginosa* DinB protein (DinB_{Pa}). Our results indicate that DinB_{Pa} is a distributive DNA polymerase that lacks intrinsic proofreading activity in vitro. Modest overexpression of DinB_{Pa} from a plasmid conferred a mutator phenotype in both *Escherichia coli* and *P. aeruginosa*. An examination of this mutator phenotype indicated that DinB_{Pa} has a propensity to promote C→A transversions and –1 frameshift mutations within poly(dGMP) and poly(dAMP) runs. The characterization of *lexA*⁺ and Δ *lexA::aacC1* *P. aeruginosa* strains, together with in vitro DNA binding assays utilizing cell extracts or purified *P. aeruginosa* LexA protein (LexA_{Pa}), indicated that the transcription of the *dinB* gene is regulated as part of an SOS-like response. The deletion of the *dinB*_{Pa} gene sensitized *P. aeruginosa* to nitrofurazone and 4-nitroquinoline-1-oxide, consistent with a role for DinB_{Pa} in translesion DNA synthesis over N²-dG adducts. Finally, *P. aeruginosa* exhibited a UV-inducible mutator phenotype that was independent of *dinB*_{Pa} function and instead required *polA* and *polC*, which encode DNA polymerase I and the second DNA polymerase III enzyme, respectively. Possible roles of the *P. aeruginosa* *dinB*, *polA*, and *polC* gene products in mutagenesis are discussed.

Various endogenous and exogenous agents can damage DNA. To guard against this damage, organisms possess various mechanisms devoted to DNA repair, including base excision repair, nucleotide excision repair, mismatch repair (MMR), and homologous recombination (reviewed in reference 22). Despite the remarkable proficiency with which these repair functions identify and correct damaged DNA, lesions sometimes evade repair and must therefore be dealt with during DNA replication. In these cases, the DNA lesion is generally tolerated via postreplicative gap repair or translesion DNA synthesis (TLS). Postreplicative gap repair refers to a class of mechanisms in which the replication machinery utilizes the information in the nascent daughter strand to replicate past the lesion in a high-fidelity manner (reviewed in references 13, 20, and 22). In contrast, TLS involves direct replication over the site(s) of damage (reviewed in references 20, 22, and 56). Since replicative DNA polymerases (Pols) are unable to efficiently extend imperfect primer/template termini, TLS in general cannot be catalyzed by these Pols and is therefore dependent upon one or more specialized Pols capable of replicating

over damaged DNA bases. Most Pols capable of participating in TLS belong to the Y family of DNA polymerases (reviewed in references 19, 21, 45, and 56). Y family Pols are typically distributive, lack proofreading activities, and possess fidelities lower than those of well-studied replicative Pols. Although Y family Pols, such as the human Pol η (XP-V Pol), can replicate UV-induced DNA lesions with relatively high fidelity (reviewed in references 29, 30, 41, 42, 48, and 64), many replication-blocking lesions are noncoding. Thus, most TLS is inherently error prone.

As a consequence of its role in damage tolerance, TLS serves as a major pathway for mutagenesis. For example, following irradiation with UV light or exposure to certain chemical carcinogens, most induced mutagenesis in *Escherichia coli* depends on the *umuDC*-encoded Pol V (32, 52). *E. coli* Pol V is regulated as part of the SOS response. In this response, the LexA protein represses the transcription of roughly 40 different genes, including *umuDC* (12, 22). Following replication-blocking DNA damage, the *E. coli* RecA protein facilitates the autodigestion of LexA, leading to the transcriptional derepression of the LexA-regulated genes (38). TLS is also believed to significantly contribute to mutagenesis in eukaryotes (21, 22, 24). Furthermore, error-prone replication by various low-fidelity Pols is reported to contribute to immunoglobulin diversity via somatic hypermutation (51).

A capacity for DNA damage tolerance and repair is important for ensuring high-fidelity replication of the genetic mate-

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rial in all organisms. In addition, recent data suggest that DNA repair and mutagenesis may also play important roles in pathogenesis. For example, for *Salmonella enterica* and *Listeria monocytogenes*, the ability to repair DNA damage appears to be important for the initial stages of infection (8–10, 57, 58, 68). For *Helicobacter pylori*, the *mutS* gene product plays an important role in pathogenesis by protecting this pathogen from oxidative stress: *mutS* mutants of *H. pylori* exhibit reduced colonization in a mouse model of infection, presumably due to their inability to repair oxidative DNA damage (62). Recently, a striking correlation between mutagenesis and the persistence of *Pseudomonas aeruginosa* was reported (46). *P. aeruginosa* is a human opportunistic pathogen that chronically infects the lungs of cystic fibrosis (CF) patients and is the leading cause of morbidity and mortality of people afflicted with this disease. Oliver et al. (46) reported that, although *P. aeruginosa* isolates recovered from individuals with acute infections displayed normal low-level spontaneous mutation frequencies, roughly 20% of the isolates recovered from CF patients suffering from chronic *P. aeruginosa* infections displayed a hypermutable phenotype. This hypermutability resulted from a loss of MMR and correlated with an increased resistance of the *P. aeruginosa* strains to various commonly used antimicrobial agents (46).

P. aeruginosa isolates recovered from cystic fibrosis patients were reported to bear mutations in *mucA* that impair its function (40). The MucA protein of *P. aeruginosa* acts as an anti- σ factor to regulate the expression of the exopolysaccharide alginate (40, 43). The finding that *P. aeruginosa* isolates recovered from CF patients possessed mutations inactivating *mucA* suggests that the *mucA* allele represents a “hot spot” for mutagenesis that confers an advantage in persistence. Nucleotide sequence analysis of the *mucA* allele in the different mucoid *P. aeruginosa* isolates indicated that most inactivating mutations corresponded to a –1 frameshift mutation within a run of five deoxyguanylic acid residues (40, 43, 49). Taken together, these results suggest a critically important role for mutagenesis in the persistence of *P. aeruginosa* infections. However, very little is known about how *P. aeruginosa* regulates TLS and mutagenesis.

As part of an effort to understand the role of Y family DNA Pols in mutagenesis in *P. aeruginosa*, particularly frameshift mutagenesis, we have cloned the *dinB* gene of *P. aeruginosa* and utilized a combination of genetic and biochemical approaches to characterize the recombinant *P. aeruginosa* DinB protein (DinB_{Pa}). Our results discussed below indicate that DinB_{Pa} is a distributive DNA polymerase that is apparently devoid of an intrinsic proofreading activity. Modest overexpression of DinB_{Pa} from a plasmid conferred a mutator phenotype in both *E. coli* and *P. aeruginosa*. Careful examination of this mutator phenotype indicated that DinB_{Pa} had a propensity to promote C→A transversions and –1 frameshift mutations within poly(dGMP) and poly(dAMP) runs. The characterization of *lexA*⁺ and Δ *lexA::aacC1* *P. aeruginosa* strains indicated that the transcription of the *dinB* gene of *P. aeruginosa* was regulated as part of an SOS-like response. Moreover, the deletion of the *dinB* allele sensitized *P. aeruginosa* to the DNA-damaging agents nitrofurazone (NFZ) and 4-nitroquinoline 1-oxide (4-NQO), suggesting that DinB_{Pa} plays an important role in damage-induced TLS. Finally, *P. aeruginosa* dis-

played a modest UV-induced mutator phenotype that was independent of *dinB* function and instead required the *polA* and *polC* genes, which encode DNA polymerase I and the second DNA polymerase III enzyme, respectively. Possible roles of *P. aeruginosa* DNA polymerase I (*polA*), the second DNA polymerase III (*polC*), and DNA polymerase IV (*dinB*) in DNA mutagenesis are discussed.

MATERIALS AND METHODS

Bacteriological techniques. The *E. coli* and *P. aeruginosa* strains used in this study are described in Table 1. Strains MPAO1 and MPA50455 (Δ *polA::ISphoA/hah*), MPA17464 (Δ *polB::ISlacZ/hah*), and MPA34086 (Δ *polC::ISphoA/hah*) were obtained from the University of Washington Genome Center (27). *ISphoA/hah* and *ISlacZ/hah* transposon insertion within the *polA*, *polB*, or *polC* gene of *P. aeruginosa* was confirmed by diagnostic PCR using a protocol provided by Michael Jacobs of the University of Washington Genome Center. All strains were routinely grown in Luria-Bertani (LB) medium. When necessary, the following antibiotics were used at the indicated concentrations with *E. coli* strains: ampicillin, 150 μ g/ml; kanamycin, 60 μ g/ml; and rifampin (Rif), 100 μ g/ml. When required, gentamicin and rifampin were each used at 100 μ g/ml with *P. aeruginosa* strains, while tetracycline was used at 60 μ g/ml, and carbenicillin was used at 250 μ g/ml.

The plasmid DNAs used in this study are described in Table 1. The *dinB* gene (including its native promoter) of *P. aeruginosa* was PCR amplified from *P. aeruginosa* PAO1 genomic DNA using primers *dinB1* (5'-CGG GAT CCG AGT TCC ATC CGG TTC ACG-3') and *dinB2* (5'-CCC AAG CTT GGG ACG CCG TGC TGA AGG CC-3'). The amplified DNA fragment was digested with BamHI and HindIII and cloned into pWSK29, resulting in pHL6. For the expression of DinB_{Pa}, a PCR amplification of plasmid pHL6 with primers *dinB4* (5'-GGA ATT CCA TAT GCG GAA AAT CAT CCA TAT AGA CTG-3') and *dinB5* (5'-CCC AAG CTT GAA CAA CCT GAG TTG TTC GT-3') was performed. The fragment was digested with NdeI and HindIII and cloned into pET29a (Novagen), resulting in plasmid pHL8, which was used for the overproduction of the hexahistidine-tagged, wild-type DinB_{Pa}. Three individual *dinB* missense mutations (D8A [aspartic acid position 8 changed to alanine], R49A, and D103A) were constructed in both pHL6 and pHL8 by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) (Table 1).

The *lexA* gene was PCR amplified from *P. aeruginosa* PAO1 genomic DNA using primers *lexA5* (5'-CCC AAG CTT GCG CCG GAT CAC GC-3') and *lexA6* (5'-CGG GAT CCA GGC GAC GAC ATG CA-3'). The resulting product was digested with HindIII and BamHI and cloned into the vector pET29a for expression with a C-terminally hexahistidine-tagged *P. aeruginosa* LexA protein (LexA_{Pa}), resulting in pAR88.

Construction of Δ *lexA::aacC1* and Δ *dinB::aacC1* *P. aeruginosa* strains. Standard reverse genetic techniques (26, 50) were used for the construction of *P. aeruginosa* *dinB*- and *lexA*-null mutants. For the Δ *dinB* mutant, plasmid pHL6 was digested with BamHI and HindIII and the *dinB* fragment was subcloned into pEX18Ap (26), resulting in pHL13. An ~200-bp fragment within the *dinB* coding sequence was removed by SalI cleavage of pHL13 and replaced with a gentamicin resistance cassette derived from SalI cleavage of pMS266 (5), generating pHL16. *P. aeruginosa* WFP334 (Δ *dinB::aacC1*) was generated using standard mating and sucrose selection gene replacement strategies (26, 39) of wild-type *dinB* in *P. aeruginosa* PAO1 with Δ *dinB::aacC1* from pHL16. A similar strategy was used to generate the *lexA::aacC1* mutant, WFP340. The *lexA* gene was amplified from PAO1 genomic DNA using primers *lexA1* (5'-CGG GAT CCG CAG GAG GTC CTC CAG GGT-3') and *lexA2* (5'-CCC AAG CTT TAT TCA GGC TCT GTG CTT GGC CC-3'), digested with BamHI and HindIII, and cloned into similarly digested pEX18Ap, resulting in pHL10. A gentamicin resistance cassette derived from SphI cleavage of pMS266 was cloned into the SphI site of pHL10, within the *lexA* coding sequence, generating pHL11. *P. aeruginosa* WFP340 (Δ *lexA::aacC1*) was generated using standard mating and sucrose selection gene replacement strategies (26, 39) of wild-type *lexA* in *P. aeruginosa* PAO1 with Δ *lexA::aacC1* from pHL11. The *sulA* gene is located immediately downstream of *lexA*; the expression of *sulA* may be affected by the *lexA::aacC1* allele, as we did not have to disrupt *sulA* prior to constructing the Δ *lexA* strain.

Purification of *P. aeruginosa* and *E. coli* DinB proteins. The *P. aeruginosa* wild-type DinB protein was overproduced using *E. coli* BL21(DE3) harboring pHL8 grown at 30°C in LB supplemented with ampicillin. When the culture reached an optical density at 595 nm (OD₅₉₅) of 0.8, IPTG (isopropyl- β -D-

TABLE 1. Bacterial strains and plasmid DNAs used in this study

Strain ^a or plasmid	Relevant genotype or characteristic ^b	Source or construction ^c
Strains		
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR1</i> (r _K ⁻ m _K ⁺) <i>glnV44 thi-1 recA1 gyrA relA</i> Δ (<i>lacZYA-argF</i>)U169 <i>deoR</i> (ϕ 80 <i>dlac</i> Δ [<i>lacZ</i>]M15)	Laboratory stock
BL21(DE3)	<i>lon ompT</i> (λ DE3)	Novagen
JM109(DE3)	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1</i> Δ (<i>lac-proAB</i>) e14 ⁻ (McrA ⁻) (λ DE3) [F' <i>traD36 proA⁺ proB⁺ lacI^q</i> Δ M15]	Promega
AB1157	<i>thr-1 ara-14 leuB6</i> Δ (<i>gpt-proA</i>)62 <i>lacY1 tsx-33 glnV44 galK2 hisG4</i> (Oc) <i>rfbD1 mgl-51 rpsL31 kdgK51 mtl-1 argE3</i> (Oc) <i>thi-1</i>	Laboratory stock
CC101	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (TAG $\rightarrow$ GAG) <i>proB⁺</i>]	15
CC102	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (GGG $\rightarrow$ GAG) <i>proB⁺</i>]	15
CC103	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (CAG $\rightarrow$ GAG) <i>proB⁺</i>]	15
CC104	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (GCG $\rightarrow$ GAG) <i>proB⁺</i>]	15
CC105	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (GTG $\rightarrow$ GAG) <i>proB⁺</i>]	15
CC106	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (AAG $\rightarrow$ GAG) <i>proB⁺</i>]	15
CC107	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (6G $\rightarrow$ 7G) <i>proB⁺</i>]	14
CC108	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (6G $\rightarrow$ 5G) <i>proB⁺</i>]	14
CC110	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (6A $\rightarrow$ 7A) <i>proB⁺</i>]	14
CC111	<i>ara</i> Δ (<i>lac-proB</i>) [F <i>lacI373 lacZ</i> (7A $\rightarrow$ 6A) <i>proB⁺</i>]	14
RW118	<i>thr-1 araD139</i> Δ (<i>gpt-proA</i>)62 <i>lacY1 tsx-33 supE44 galK2 hisG4</i> (Oc) <i>rpsL31 xyl-5 mtl-1 argE3</i> (Oc) <i>thi-1 sulA211</i>	25
RM137	RW118 but Δ (<i>dinB-yafN</i>)::kan	This work
<i>P. aeruginosa</i>		
PAO1	Prototroph	Laboratory stock
WFPA340	PAO1 with Δ <i>lexA</i> :: <i>aacC1</i> (Gen ^r)	This work
WFPA334	PAO1 with Δ <i>dinB</i> :: <i>aacC1</i> (Gen ^r)	This work
MPAO1	Prototroph	27
MPA50455	MPAO1 with Δ <i>polA</i> ::IS <i>phoA</i> /hah (Tet ^r)	27
MPA17464	MPAO1 with Δ <i>polB</i> ::IS <i>lacZ</i> /hah (Tet ^r)	27
MPA34086	MPAO1 with Δ <i>polC</i> ::IS <i>phoA</i> /hah (Tet ^r)	27
Plasmids		
pWSK29	Ap ^r ; pSC101 origin, general cloning vector	63
pHL6	Ap ^r ; pWSK29 bearing <i>P. aeruginosa</i> <i>dinB⁺</i>	This work
pHL6-D8A	Ap ^r ; pHL6 bearing <i>P. aeruginosa</i> <i>dinB</i> (D8A)	This work
pHL6-R49A	Ap ^r ; pHL6 bearing <i>P. aeruginosa</i> <i>dinB</i> (R49A)	This work
pHL6-D103A	Ap ^r ; pHL6 bearing <i>P. aeruginosa</i> <i>dinB</i> (D103A)	This work
pET29a	Km ^r ; pET vector used for overexpression of <i>P. aeruginosa</i> DinB, encodes C-terminal His ₆ tag	Novagen
pHL8	Km ^r ; pET29a bearing <i>P. aeruginosa</i> <i>dinB⁺</i>	This work
pHL8-D8A	Km ^r ; pHL8 bearing <i>P. aeruginosa</i> <i>dinB</i> (D8A)	This work
pHL8-R49A	Km ^r ; pHL8 bearing <i>P. aeruginosa</i> <i>dinB</i> (R49A)	This work
pHL8-D103A	Km ^r ; pHL8 bearing <i>P. aeruginosa</i> <i>dinB</i> (D103A)	This work
pAR88	Km ^r ; pET29a bearing <i>P. aeruginosa</i> <i>lexA⁺</i>	This work
pEX18Ap	Ap ^r ; suicide replacement vector containing <i>B. subtilis</i> <i>sacB</i> gene	26
pHL13	Ap ^r ; pEX18Ap bearing <i>P. aeruginosa</i> <i>dinB⁺</i>	This work
pMS266	Ap ^r , Gen ^r ; contains a 1.1-kb <i>aacC1</i> gentamicin resistance cassette	5
pHL16	Ap ^r , Gen ^r ; pHL13 bearing <i>P. aeruginosa</i> Δ <i>dinB</i> :: <i>aacC1</i> (Gen ^r)	This work
pHL10	Ap ^r ; pEX18Ap bearing <i>P. aeruginosa</i> <i>lexA⁺</i>	This work
pHL11	Ap ^r , Gen ^r ; pHL10 bearing <i>P. aeruginosa</i> Δ <i>lexA</i> :: <i>aacC1</i> (Gen ^r)	This work
pUCP20T	Ap ^r , cloning vector that replicates in both <i>P. aeruginosa</i> and <i>E. coli</i>	Laboratory stock
pAR101	Ap ^r , pUCP20T bearing <i>dinB_{Pa}⁺</i>	This work

^a *E. coli* strain CC109 was not used due to the fact that the reversion of its mutant *lacZ* allele was reported to be influenced by multiple factors (J. Miller, personal communication).

^b Ap, ampicillin; Gen, gentamicin; Km, kanamycin; Tet, tetracycline.

^c *E. coli* strains, *P. aeruginosa* strains, and plasmid DNA constructions are described in detail in Materials and Methods.

thiogalactopyranoside) (Promega) was added to 50 μ M and growth was continued for another 3 h. Cells were harvested by centrifugation, resuspended in buffer H (50 mM NaPO₄ [pH 6.8], 500 mM NaCl, 10% glycerol, 20 mM 2-mercaptoethanol, and 1 mM imidazole), and lysed by double passage through a chilled French press. The soluble fraction was clarified by centrifugation at 4°C prior to chromatography. The extract containing the C-terminally hexahistidine-tagged DinB_{Pa} protein was first applied to a 5-ml Ni-nitrilotriacetic acid (NTA) agarose column (QIAGEN). Bound protein was eluted with buffer H containing 50 mM EDTA. Fractions containing DinB_{Pa}, identified by sodium dodecyl sul-

fate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled and dialyzed against buffer A (25 mM Tris-HCl [pH 7.0], 10% glycerol, and 20 mM 2-mercaptoethanol) containing 25 mM NaCl prior to chromatography using a 1-ml HiTrap heparin column (GE Healthcare) equilibrated in the same buffer. Bound protein was eluted from the heparin column by using a linear gradient of 2.5% to 100% buffer B (25 mM Tris-HCl [pH 7.0], 10% glycerol, 20 mM 2-mercaptoethanol, and 1 M NaCl). Fractions containing DinB_{Pa} were pooled, dialyzed, and applied to a Mono S HR 5/5 column (GE Healthcare) equilibrated in buffer A containing 25 mM NaCl. Bound protein was eluted using a linear gradient of

FIG. 1. In vitro DinB_{Pa} DNA polymerase activity. (A) In vitro primer extension assays were performed as described in Materials and Methods using the 20-mer/100-mer DNA template, a schematic of which is shown at the top of panel A. Specific conditions for each assay are indicated at the bottom of the figure. (B) Assays were as described for panel A but used either the 20-mer primer alone (lanes 1 and 2) or the 20-mer primer annealed to M13mp18 ssDNA (NEB) (see schematic at the top of panel B). Conditions for each assay are indicated. See the text for further details.

2.5% to 100% buffer B. The purity of the final material was judged to be >95% based on densitometric scanning of Coomassie-stained SDS-PAGE gels. DinB(D8A)_{Pa} and DinB(R49A)_{Pa} were purified using the same approach. The DinB(D103A)_{Pa} mutant protein became insoluble during its purification. Purified *E. coli* DinB protein bearing a C-terminal hexahistidine tag was a generous gift from Daniel Jarosz and Graham Walker (Massachusetts Institute of Technology).

In vitro primer extension assay. Replication assays (10 μl) consisted of a 5'-[³²P]ATP radiolabeled (using T4 polynucleotide kinase; Promega) synthetic 20-mer (5'-AC GCC TGT AGC ATT CCA CAG-3')/100-mer (5'-AA TCC CAT ACA GAA AAT TCA TTT ACT AAC GTC TGG AAA CTC GAC AAA ACT TTA GAT CGA AAC GCT AAC TAT GAG GGG TGT CTG TGG AAT GCT ACA GGC GT-3') DNA template (gel purified) (Fig. 1A) or the same 20-mer either alone or annealed to M13mp18 single-stranded DNA (ssDNA) (Fig. 1B) at a final concentration of 5 nM in replication buffer (20 mM Tris-HCl [pH 7.5], 8 mM MgCl₂, 4% glycerol, 50 mM NaCl, 5 mM dithiothreitol, 40 μg/ml bovine serum albumin) containing 133 μM of either each individual or all four ultrapure deoxynucleoside triphosphates (dNTPs) (GE Healthcare), as indicated in the legend for Fig. 1. Reactions were initiated by the addition of DinB_{Pa} or the *E. coli* DinB protein (DinB_{Ec}) at the indicated concentrations (see the legend for Fig. 1) for 10 or 20 min at 37°C, as noted. Reactions were quenched with EDTA, heat denatured, and electrophoresed through 10% denaturing polyacrylamide gels. Radiolabeled products were visualized using a phosphorimager with a signal-intensifying phosphorimaging screen (Bio-Rad).

RNA harvest and real-time RT-PCR. Total RNA was harvested from *P. aeruginosa* strains PAO1 (*lexA*⁺) and WFP340 (*ΔlexA::aacCI*) using the Ambion Ribo-Pure kit according to the manufacturer's instructions. For experiments

investigating DNA damage-induced expression of *dinB*, cultures were grown to an OD₆₀₀ of 0.6 and then treated with mitomycin C (MMC; 1 μg/ml) for 2 h. RNA was then harvested and quantified for real-time reverse transcription-PCR (RT-PCR) analysis. Following purity confirmation by gel electrophoresis, 100 ng of this RNA was used as a template in a one-step real-time RT-PCR (TaqMan one-step RT-PCR kit) with primers (*dinBF* [5'-GGC GGT GTC GAA GCA GAT-3'] and *dinBR* [5'-CGA AAG CGG CTC CAT CAG-3']) and a fluorescent probe specific for *dinB* mRNA (*dinBP* [5'-CAT GCG ATC TTC CGC GAT TAT ACC GA-3']). Reactions were performed and analyzed using an ABI Prism 7000 sequence detection system. For each RNA sample, *dinB* mRNA levels were normalized against *rpoD* mRNA levels, which were determined using primers *rpoDF* (5'-CCT GCC GGA GGA TAT TTC C-3') and *rpoDR* (5'-GAT CCC CAT GTC GTT GAT CAT-3') and a fluorescent probe specific for *rpoD* mRNA (*rpoDP*, 5'-ATC CGG AAC AGG TGG AAG ACA TCA TCC-3'). Once normalized against *rpoD*, data were presented as a severalfold increase over wild-type (PAO1) levels of *dinB*_{Pa} mRNA, which were set equal to 1.0.

Western blot analysis. Purified hexahistidine-tagged DinB_{Pa} derived from pHL8 was used to elicit antibodies from New Zealand White rabbits (Covance Research Products). Anti-DinB_{Pa} serum was adsorbed against extracts derived from WFP334 (*ΔdinB::aacCI*) and subsequently used at a dilution of 1/10,000 as the primary antibody for the detection of DinB_{Pa} by Western blotting. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Pierce) was used as the secondary antibody at a dilution of 1/10,000. Western blotting was performed using either whole-cell extracts (Fig. 2) or cell extracts (Fig. 3), and purified hexahistidine-tagged DinB_{Pa} protein as a standard. Whole-cell extracts were prepared as described previously (55). For the preparation of cell extracts, *P. aeruginosa* strains were grown to an OD₆₀₀ of 0.6, 10 ml of each culture was

FIG. 2. Steady-state levels of DinB_{Pa}^+ , $\text{DinB}(\text{D8A})_{Pa}$, $\text{DinB}(\text{R49A})_{Pa}$, and $\text{DinB}(\text{D103A})_{Pa}$. Whole-cell extracts of *E. coli* strain FC1237 [relevant genotype, $\Delta(\text{dinB-yafN}):kan$ (54)], bearing the indicated plasmids, were prepared and separated by SDS-PAGE, and blotting was performed using anti- DinB_{Pa} antibodies as described in Materials and Methods. Lanes 1 and 2 contain 1.0 and 0.1 μg , respectively, of the purified hexahistidine-tagged DinB_{Pa} protein.

centrifuged, and the pellets were resuspended in 1.0 ml of FBG (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM MgCl_2 , 5% glycerol). Samples were subjected to sonication and centrifuged for 15 min at 14,000 rpm. Protein concentration in the supernatant fraction was quantified by the bicinchoninic acid assay. Equivalent amounts of protein from extracts were separated by 12% SDS-PAGE and analyzed by Western blotting.

EMSA. Electrophoretic mobility shift assay (EMSA) experiments were performed generally as described previously (18). DNA fragments for dinB_{Pa} EMSA were labeled by PCR amplification of *P. aeruginosa* PAO1 genomic DNA with primers *dinB1* (above) and *dinB3* (5'-CCC AAG CTT CGA GGG CGG CAT AGA AAC AGT-3') and [$\alpha\text{-}^{32}\text{P}$]dCTP following previously published protocols (3). Primers *umuprom1* (5'-GCG TCG TCG CCA GAA GG-3') and *umuprom2* (5'-CGT AAT CTG CTG CCG GTG-3') were used to amplify the 5' regulatory sequences of *E. coli umuC* using *E. coli* MG1655 genomic DNA. Cell extracts, purified LexA_{Ec} , or LexA_{Pa} was incubated with the DNA fragments in a 5- μl final volume for 30 min in binding buffer (10 mM HEPES-NaOH [pH 8.0], 10 mM Tris-HCl [pH 8.0], 5% glycerol, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol). A 6% nondenaturing polyacrylamide gel was prerun for 1 h at 50 V at 4°C with electrophoresis buffer (25 mM Tris-HCl [pH 8.0], 250 mM glycine, 1 mM EDTA). Samples were loaded and resolved overnight at 50 V at 4°C. The gel was dried and visualized using a phosphorimager with a signal-intensifying phosphorimaging screen (Bio-Rad). LexA_{Ec} was kindly provided by John Little (University of Arizona). Hexahistidine-tagged LexA_{Pa} protein derived from JM109(DE3)(pAR88) was purified using Ni-NTA agarose resin (QIAGEN). Bound protein was eluted from Ni-NTA agarose using imidazole. LexA_{Pa} purified in this fashion contained two distinct bands of approximately 26 and 14 kDa, which likely represent mature and cleaved LexA , respectively.

RESULTS

The *P. aeruginosa dinB* gene encodes a functional DNA polymerase (Pol_{Pa} IV). Based on the Cluster of Orthologous Groups of the National Center for Biotechnology Information, the *P. aeruginosa* genome contains a single ortholog of *E. coli umuC* and *dinB* designated PA0923 (53). As PA0923 resembled *E. coli dinB* more closely than it did *E. coli umuC*, it was suggested to be a *dinB* (DNA Pol IV) ortholog. The predicted *P. aeruginosa* DinB protein (DinB_{Pa}) is 342 amino acids (aa) and exhibits 49% identity with DinB_{Ec} (340 aa). Moreover, residues D8, R49, and D103, which are essential for DinB_{Ec} activity (60) are completely conserved in DinB_{Pa} . In order to determine whether DinB_{Pa} possessed an intrinsic nucleotidyl transferase activity, the C-terminally hexahistidine-tagged protein was purified to apparent homogeneity and assayed in an in vitro primer extension assay (see Materials and Methods). Using a synthetic 20-mer primer annealed to a 100-mer as a template (20/100 template) (Fig. 1A), DinB_{Pa} was able to incorporate dNTPs with efficiencies compa-

FIG. 3. Expression of *dinB* is DNA damage regulated. (A) Real-time RT-PCR analysis of *dinB* mRNA from *P. aeruginosa* strains PAO1 (lexA^+) and WFP340 ($\Delta\text{lexA}::\text{aacC1}$). PAO1+MMC represents strain PAO1 treated for 2 h with 1 $\mu\text{g}/\text{ml}$ MMC before RNA harvest. WFP340 is the mean of five replicate experiments (range, 3.05 to 11.52), and PAO1+MMC is the mean of nine replicate experiments (range, 2.35 to 10.36). All values are normalized against the levels of *rpoD* (see Materials and Methods). (B) Western blot showing DinB_{Pa} levels in *P. aeruginosa* strains PAO1 (lexA^+), WFP340 ($\Delta\text{lexA}::\text{aacC1}$), and WFP334 ($\Delta\text{dinB}::\text{aacC1}$). Cell extracts were prepared and separated by SDS-PAGE, and blotting was performed using anti- DinB_{Pa} antibodies as described in Materials and Methods. Upper panel, DinB ; lower panel, non-specific cross-reactive band used for a normalizing control. Lane 1, purified His-tagged DinB_{Pa} ; lane 2, PAO1 extract; lane 3, WFP340 extract; lane 4, PAO1+MMC extract; lane 5, WFP334 extract.

table to that observed with DinB_{Ec} (Fig. 1A, compare lanes 1 to 3 and 9 to 11). Interestingly, in assays utilizing the same 20-mer primer annealed to ssM13 viral DNA as a template, significantly higher concentrations of DinB_{Pa} were required in order to visualize polymerase activity (Fig. 1, compare lanes 3 to 5 of panel B with lanes 9 to 11 of panel A), suggesting that it was inefficient at binding to the primer-template junction if challenged with extensive single-strand DNA. This is reminiscent of reports describing the DinB_{Ec} enzyme (61). Importantly, DinB_{Pa} nucleotidyl transferase activity was dependent upon the template DNA (Fig. 1B, compare lanes 1 and 2), indicating that it was a DNA polymerase and not a terminal transferase. The absence of primer degradation in the reaction containing only DinB_{Pa} and the 20/100 DNA substrate (Fig. 1A, lane 12) suggests that DinB_{Pa} , like DinB_{Ec} (Fig. 1A, lane 4), is devoid of an intrinsic 3'-to-5' exonuclease (proofreading) activity.

The accuracy of nucleotide incorporation by DinB_{Pa} in vitro on an undamaged template was investigated qualitatively by assessing the efficiency of the template-directed incorporation of each single dNTP. Using the 20/100 as a template, DinB_{Pa} efficiently catalyzed the incorporation of dATP opposite template dTMP (Fig. 1A, lane 13). In contrast, DinB_{Pa} did not incorporate dTTP, dGTP, or dCTP opposite template dTMP (Fig. 1A, lanes 14 to 16). In the presence of all four nucleotides, DinB_{Pa} extended the primer in what appeared to be a largely distributive manner (Fig. 1A, lane 11). Similar results were observed for DinB_{Ec} (Fig. 1A, lanes 3 and 5 to 8), consistent with previous reports (60).

Amino acid substitutions at residues D8, R49, and D103 of

TABLE 2. Effect of *P. aeruginosa* *dinB* overexpression on the frequency of Rif^r in *E. coli*

AB1157 transformant ^a	<i>dinB</i> allele	Rif ^r CFU/10 ⁸ CFU ± SEM (<i>P</i> value) ^b	Increase (<i>n</i> -fold) relative to pWSK29 control
pWSK29	None	11.6 ± 8.7 (NA)	≡1.0 ^c
pHL6	<i>dinB</i> _{Pa}	54.1 ± 48.3 (0.002)	4.7
pHL6-D8A	<i>dinB</i> (D8A) _{Pa}	7.2 ± 3.4 (0.700)	0.6
pHL6-R49A	<i>dinB</i> (R49A) _{Pa}	13.2 ± 8.6 (0.340)	1.1

^a Each plasmid was transformed into *E. coli* strain AB1157. Aliquots of appropriate serial dilutions of cultures started from single colonies inoculated into 5 ml of LB-Ap and grown ~16 h were plated on LB to determine survival and on LB-Rif to assess spontaneous mutation rate. Values shown represent averages of 4 to 16 independent experiments ± the standard error of the means. Median (*m*) and range (*r*) values are as follows: for pWSK29, *m* = 8.25 and *r* = 0.87 to 29.6; for pHL6, *m* = 50.4 and *r* = 6.9 to 146.9; for pHL6-D8A, *m* = 7.1 and *r* = 3.8 to 11.0; and for pHL6-R49A, *m* = 14.1 and *r* = 2.5 to 25.0.

^b *P* values were calculated based on values for Rif^r for each *DinB*_{Pa}-expressing strain relative to the pWSK29 control strain. NA, not applicable.

^c ≡1.0, set equal to 1.0.

*DinB*_{Ec} were reported to severely impair its catalytic activity both in vitro and in vivo (60). Using site-directed mutagenesis, we replaced these corresponding residues in *DinB*_{Pa} with alanine. The *DinB*(D8A)_{Pa} and *DinB*(R49A)_{Pa} mutant proteins purified in a manner that was indistinguishable from that of the wild-type *DinB*_{Pa}. In contrast, the *DinB*(D103A)_{Pa} mutant protein became insoluble during its purification and was therefore not evaluated for in vitro activity. Although no polymerase activity was detected with the *DinB*(D8A)_{Pa} mutant protein in vitro (Fig. 1A, lanes 17 to 18), the *DinB*(R49A)_{Pa} mutant protein retained detectable, albeit significantly reduced, polymerase activity (Fig. 1A, lanes 19 to 21).

Elevated expression of *DinB*_{Pa} confers a mutator phenotype in vivo. Overexpressed levels of *DinB*_{Ec} from a plasmid were reported to increase the frequency of mutagenesis in *E. coli* as measured using a Rif^r assay (28, 34, 61, 66, 67). We therefore hypothesized that if *DinB*_{Pa} was similarly error prone, then

plasmid-expressed *DinB*_{Pa} would likewise increase the frequency of Rif^r in *E. coli*. The expression of *dinB* of *P. aeruginosa* from a low-copy-number plasmid (pHL6) conferred a 4.7-fold increase in the frequency of Rif^r relative to the same strain bearing the pWSK29 control vector (Table 2). In contrast, neither plasmid-expressed *DinB*(D8A)_{Pa} nor *DinB*(R49A)_{Pa} enhanced the frequency of Rif^r (Table 2). Given that the D8A and R49A mutants were expressed at steady-state levels comparable to that of the wild-type protein (Fig. 2), these findings indicate that the modest Rif^r mutator phenotype conferred by ectopically expressed *DinB*_{Pa} was attributable to its intrinsic DNA polymerase activity. We also intended to test whether the *DinB*(D103A)_{Pa} mutant protein was impaired for the Rif^r mutator phenotype, but we determined that this mutant protein was present at a reduced steady-state level compared to that of the wild-type *DinB*_{Pa} or to those of the D8A and R49A mutants (Fig. 2). This finding, taken together with our difficulties in purifying *DinB*(D103A)_{Pa}, suggested that this mutant protein was misfolded when expressed in *E. coli*. Therefore, *DinB*(D103A)_{Pa} was not examined using this approach.

In order to determine the spectrum of mutations made by *DinB*_{Pa}, we utilized a *lacZ*→Lac⁺ reversion assay developed by Cupples and Miller (14, 15). This assay takes advantage of a series of isogenic strains bearing different mutant *lacZ* alleles. The ability of these strains to grow on minimal medium containing lactose as a sole carbon source requires that the *lacZ* allele (either a base substitution or a frameshift mutation) undergo a “true” reversion, as no other mutation will restore the Lac⁺ phenotype (14, 15). By comparing frequencies of *lacZ*→Lac⁺ reversion for transformants of these reporter strains bearing either the pWSK29 control vector or the pHL6, which expresses *DinB*_{Pa}⁺, we were able to determine the spectrum of mutations generated by ectopic expression of *DinB*_{Pa}. As summarized in Table 3, *DinB*_{Pa} clearly favored −1 frameshift mutations: the frequency of Lac⁺ reversion was increased 339- and 3-fold in the CC108 (*lacZ*[6G→5G]) and the CC111

TABLE 3. Abilities of plasmid-expressed *DinB*_{Pa}⁺, *DinB*(D8A)_{Pa}, and *DinB*(R49A)_{Pa} mutants to influence frequencies of *lacZ*→Lac⁺ reversion of different *lacZ* alleles

Strain ^a	Base change ^a	Lac ⁺ revertants/10 ⁷ CFU ± SEM (<i>P</i> value) with ^b :			
		pWSK29 (vector control)	pHL6 (<i>DinB</i> _{Pa} ⁺)	pHL6-D8A [<i>DinB</i> (D8A) _{Pa}]	pHL6-R49A [<i>DinB</i> (R49A) _{Pa}]
CC101	TAG→GAG	<0.05	<0.04 (NA)	ND	ND
CC102	GGG→GAG	<0.08	<0.14 (NA)	ND	ND
CC103	CAG→GAG	<0.12	<0.07 (NA)	ND	ND
CC104	GCG→GAG	0.10 ± 0.00	11.70 ± 7.50 (0.056)	0.30 ± 0.20 (0.130)	5.30 ± 2.00 (0.012)
CC105	GTG→GAG	0.17 ± 0.15	0.31 ± 0.29 (0.500)	ND	ND
CC106	AAG→GAG	<0.10	<0.10 (NA)	ND	ND
CC107	6G→7G	0.10 ± 0.17	<0.53 (NA)	ND	ND
CC108	6G→5G	2.87 ± 0.21	972.30 ± 209.90 (0.001)	30.4 ± 22.00 (0.099)	54.7 ± 8.90 (0.005)
CC110	6A→7A	0.13 ± 0.15	0.23 ± 0.12 (0.390)	ND	ND
CC111	7A→6A	2.17 ± 0.67	6.57 ± 2.35 (0.036)	ND	ND

^a See Table 1 for a complete description of the strains and base changes in the different *lacZ* alleles. For strains CC101 to CC106, the specific base pair changes required for *lacZ*→Lac⁺ reversion are presented within the context of their codon sequences; the base that must undergo reversion in order to yield a Lac⁺ phenotype is underlined.

^b *lacZ*→Lac⁺ reversion frequencies were measured using cultures inoculated with single colonies and grown overnight as described previously (33). Values shown represent the averages of at least three independent determinations ± the standard error of the means. Parenthetical values indicate *P* values (Student's *t* test) for the difference between the frequencies of *lacZ*→Lac⁺ reversion for the indicated plasmid-bearing strain relative to the same strain bearing the pWSK29 control plasmid. Median (*m*) and range (*r*) values are as follows: for CC104(pWSK29), *m* = 0.1 and *r* = 0.1 to 0.1; for CC104(pHL6), *m* = 10.0 and *r* = 5.4 to 20; for CC104(pHL6-D8A), *m* = 0.3 and *r* = 0.1 to 0.4; for CC104(pHL6-R49A), *m* = 4.6 and *r* = 3.7 to 7.6; for CC108(pWSK29), *m* = 2.8 and *r* = 2.7 to 3.1; for CC108(pHL6), *m* = 867.0 and *r* = 836.0 to 1,214.0; for CC108(pHL6-D8A), *m* = 25.2 and *r* = 11.2 to 54.7; for CC108(pHL6-R49A), *m* = 51.4 and *r* = 48.1 to 64.7; for CC111(pWSK29) *m* = 2.5 and *r* = 1.4 to 2.6; and for CC111(pHL6), *m* = 6.4 and *r* = 4.3 to 9.0. NA, not applicable; ND, not determined.

TABLE 4. Effect of *P. aeruginosa* *dinB* overexpression on the frequency of Rif^r in *P. aeruginosa*

<i>P. aeruginosa</i> strain ^a	Relevant genotype	Plasmid	Rif ^r CFU/10 ⁸ CFU $\pm$ SEM (<i>P</i> value) ^b	Increase (<i>n</i> -fold) relative to respective PAO1 control
PAO1 (control)	<i>lexA</i> ⁺ <i>dinB</i> ⁺	pUCP20T (control)	2.6 $\pm$ 1.2 (NA)	$\equiv$ 1.0 ^c
PAO1	<i>lexA</i> ⁺ <i>dinB</i> ⁺	pAR101 (<i>dinB</i> _{Pa} ⁺)	6.7 $\pm$ 2.7 (0.01)	2.6
PAO1 (control)	<i>lexA</i> ⁺ <i>dinB</i> ⁺	None	2.3 $\pm$ 0.8 (NA)	$\equiv$ 1.0 ^c
WFPA340	Δ <i>lexA::aacC1</i> <i>dinB</i> ⁺	None	2.6 $\pm$ 2.5 (0.81)	1.1

^a *P. aeruginosa* strains are described in Table 1.

^b Mutation frequencies were measured using cultures inoculated with single colonies and grown overnight. Values presented for spontaneous mutation frequencies represent the average from at least five independent experiments $\pm$ the standard error of the means. Differences between respective spontaneous mutation frequencies of PAO1, bearing plasmid pUCP20T, relative to plasmid pAR101 were statistically significant (*P* value of 0.01), while those between PAO1 and WFPA340 were not statistically significant (*P* value of 0.81) at the 95% confidence level based on the Student *t* test. Median (*m*) and range (*r*) values for spontaneous mutation frequencies are as follows: for PAO1 (pUCP20T), *m* = 2.9 and *r* = 0.6 to 3.7; for PAO1 (pAR101), *m* = 7.0 and *r* = 3.3 to 9.9; for PAO1, *m* = 2.5 and *r* = 1.0 to 3.0; and for WFPA340, *m* = 1.4 and *r* = 0.6 to 6.7. NA, not applicable.

^c Values represent the increase (*n*-fold) in mutation frequency either of PAO1(pAR101) relative to PAO1(pUCP20T) or of PAO1 relative to WFPA340. $\equiv$ 1.0, set equal to 1.0.

(*lacZ*[7A→6A]) strains, respectively, relative to the pWSK29 control (*P* values of 0.001 and 0.036, respectively). Furthermore, DinB_{Pa} also increased by 117-fold the frequency of Lac⁺ reversion in the CC104 (*lacZ*[GCG→GAG]) strain (*P* value 0.056). Frequencies of Lac⁺ reversion observed with the other reporter strains bearing the DinB_{Pa}-expressing plasmid (pHL6) were comparable to those of the respective strains bearing pWSK29 (Table 3).

We next asked whether the DNA polymerase activity of DinB_{Pa} was necessary for increased *lacZ*→Lac⁺ reversion. Due to the fact that DinB_{Pa}⁺ enhanced *lacZ*→Lac⁺ reversion in the CC104 and CC108 strains more strongly than in the other strains examined (Table 3), we focused on these two strains for this experiment. As summarized in Table 3, the D8A mutant was unable to significantly alter the frequency of *lacZ*→Lac⁺ reversion in both the CC104 and the CC108 strains. Despite its inability to confer a mutator phenotype in *E. coli* as measured by Rif^r (Table 2), the R49A mutant nonetheless enhanced the frequency of *lacZ*→Lac⁺ reversion in both the CC104 and CC108 strains by factors of 53- and 19-fold, respectively: these increases were statistically significant (*P* values of 0.012 and 0.005, respectively) and are consistent with the R49A mutant retaining partial catalytic activity (Fig. 1A, lanes 19 to 21). Taken together, these findings indicate that DinB_{Pa} is capable of catalyzing both misinsertion and frame-shift mutations.

As DinB_{Pa} has the ability to confer a mutator phenotype in *E. coli*, we next investigated whether DinB contributes to mutagenesis in *P. aeruginosa*. To do this, we measured the frequency of Rif^r of PAO1 bearing either a control plasmid (pUCP20T) or a plasmid directing expression of *dinB*_{Pa} (pAR101). As shown in Table 4, the overexpression of *dinB*_{Pa} resulted in a 2.6-fold increase in mutation frequency of PAO1.

LexA-mediated repression of *dinB*_{Pa}. As DinB_{Pa} is able to contribute to mutagenesis in *P. aeruginosa* when overexpressed (Table 4), we were interested in investigating the regulation of *dinB*_{Pa}. In *E. coli* and related bacteria, TLS is largely regulated by the SOS response (19, 21, 56). As part of this response, the LexA protein acts to repress the transcription of more than 40 different genes by binding to DNA sequences overlapping their promoter regions (12, 38). Following replication-blocking DNA damage, the LexA protein undergoes a RecA-facilitated autodigestion that serves to inactivate its DNA binding activity,

leading to the transcriptional derepression of the SOS-regulated genes, including the three *E. coli* DNA Pols involved in TLS (Pol II, Pol IV, and Pol V). In an attempt to understand the regulation of *dinB*_{Pa}, we constructed a PAO1 Δ *lexA::aacC1* mutant (WFPA340) by inserting a gentamicin resistance cassette (*aacC1*) within *lexA* coding sequences (see Materials and Methods). Real-time RT-PCR analysis was then performed with RNA derived from the PAO1 (*lexA*⁺) and the WFPA340 (Δ *lexA::aacC1*) strains using a *dinB*-specific probe and primers. Real-time RT-PCR experiments showed a 5.5-fold increase in *dinB* mRNA levels in WFPA340 compared with PAO1 *dinB* mRNA levels (Fig. 3A). We also tested whether DNA damage caused a similar up-regulation of *dinB* mRNA. Real-time RT-PCR analysis of MMC-treated PAO1 cells showed an increase in *dinB* mRNA levels similar to that of WFPA340 (Fig. 3A). We next addressed the effect of the Δ *lexA::aacC1* allele on DinB_{Pa} protein levels. Cell-free protein extracts of PAO1 and WFPA340 were prepared, separated by SDS-PAGE, and processed by Western blotting using anti-DinB_{Pa} antibodies. A significant increase in DinB_{Pa} protein levels was observed in WFPA340 compared with PAO1 (Fig. 3B, compare lanes 2 and 3). When extracts were made from cultures of PAO1 treated with MMC, an increase in the steady-state levels of DinB_{Pa} was observed. These data support the hypothesis that LexA_{Pa} acts to repress *dinB*_{Pa} transcription and suggest that, like in *E. coli*, the transcription of *dinB*_{Pa} is regulated as part of an SOS-like response to DNA damage.

We next wished to determine whether LexA-dependent repression of *dinB*_{Pa} was due to a direct interaction of LexA_{Pa} with *dinB*. EMSAs were used in all DNA binding studies (Fig. 4). First, we examined whether purified LexA_{Ec} would bind a radiolabeled *dinB*_{Pa} promoter DNA. A *dinB*_{Pa} fragment extending 450 bp upstream of the coding sequence and containing a putative LexA binding site (5'-CTGT-N₈-ACAG-3') located ~350 bp upstream of the predicted *dinB* start codon was used in these studies (p*dinB*_{Pa}). At all concentrations tested, LexA_{Ec} was unable to bind to *dinB*_{Pa} sequences (Fig. 4A), despite the fact that the LexA_{Ec} preparation readily recognized *umuDC*_{Ec} target DNA (Fig. 4B, lanes 2 to 3). However, a protein(s) present in extracts derived from *P. aeruginosa* PAO1 bound to the *umuDC*_{Ec} fragment (Fig. 4B, lane 4). Data supporting the hypothesis that this protein is LexA_{Pa} are (i) that no protein-DNA complex was formed when WFPA340

FIG. 4. LexA_{Pa} and LexA_{Ec} DNA binding studies. (A) A radiolabeled *dinB*_{Pa} promoter DNA fragment (*pdinB*_{Pa}) was left untreated (lane 1) or incubated with increasing amounts of purified LexA_{Ec} (2.5 nM, 10 nM, 50 nM, 100 nM; lanes 2 to 5, respectively). (B) Radiolabeled *umuDC*_{Ec} promoter DNA (*pumuDC*_{Ec}) was incubated with the following extracts or purified LexA_{Ec} samples: lane 1, no protein; lane 2, 50 nM; lane 3, 100 nM; lane 4, 10 μ g extract; lane 5, 10 μ g extract. (C) A radiolabeled *pdinB*_{Pa} fragment was incubated with the following: lane 1, no protein; lane 2 to 4, 5.0 μ g extract; lane 5 to 7, 10 μ g extract; lane 8, no protein; lane 9 to 14, increasing amounts of LexA_{Pa} (100 ng, 500 ng, 750 ng, 1,000 ng, 1,500 ng, and 2,000 ng, respectively).

(Δ LexA::*aacC1*) extracts were tested (Fig. 4B, lane 5), while protein-DNA complexes were readily observed when wild-type *P. aeruginosa* extracts were tested with a radiolabeled *dinB*_{Pa} fragment (Fig. 4C, lanes 2 and 5), and (ii) that no binding was observed when the extracts were prepared from PAO1 cells treated with MMC (Fig. 4C, lanes 3 and 6) or from extracts prepared from Δ LexA::*aacC1* cells (Fig. 4C, lanes 4 and 7).

To verify the above findings, *P. aeruginosa* *lexA* was cloned and a hexahistidine-tagged form of LexA_{Pa} was generated (see Materials and Methods). When LexA_{Pa} was incubated with the ³²P-radiolabeled *dinB* promoter DNA, a protein-DNA complex with a mobility similar to that seen with *P. aeruginosa* extracts was observed (Fig. 4C, lanes 9 to 14), indicating that purified LexA_{Pa} binds to *dinB*_{Pa} promoter sequences. This finding, when combined with the real time RT-PCR and DinB_{Pa} Western blot analysis (Fig. 3), strongly implies a role for LexA_{Pa} in directly repressing *dinB*, likely as part of an SOS-like response.

In light of our finding that the expression of *dinB*_{Pa} was LexA-regulated (Fig. 3 and 4), we next asked whether the disruption of *lexA*_{Pa} (*lexA*::*aacC1*), which results in elevated steady-state levels of DinB_{Pa} (Fig. 3B), conferred a mutator

phenotype in *P. aeruginosa*. As shown in Table 4, the mutation frequency of *P. aeruginosa* strain WFP340 (Δ LexA::*aacC1*) was indistinguishable from that of its isogenic *lexA*⁺ partner. Taken together, results discussed above indicate that DinB_{Pa} can contribute to mutagenesis when expressed at an elevated level (Table 4) and suggest that the inactivation of the LexA_{Pa} repressor alone is insufficient for DinB_{Pa} to contribute to mutagenesis under the conditions examined thus far.

Role of *dinB*_{Pa} in DNA damage-induced mutagenesis. We next asked whether DinB functions in induced mutagenesis in *P. aeruginosa*. Based on our findings that (i) *dinB*_{Pa} expression was regulated by LexA and induced by MMC treatment (Fig. 3 and 4) and (ii) elevated levels of *dinB*_{Pa} conferred a modest mutator effect in *P. aeruginosa* (Table 4), we hypothesized that MMC treatment might confer a mutator phenotype on PAO1 that was dependent, at least in part, on DinB_{Pa}. To test this hypothesis, we treated PAO1 (*dinB*⁺) and WFP340 (Δ *dinB*::*aacC1*) with 1 μ g/ml MMC for 2 h and then washed the cells and cultured them overnight in LB prior to plating appropriate dilutions onto both LB and LB-Rif plates to determine mutation frequencies. Despite the fact that MMC treatment induced the expression of DinB_{Pa} (Fig. 3), we failed to observe an MMC-induced mutator phenotype with PAO1 (3.1 ± 1.2 [untreated] versus 2.8 ± 0.83 [MMC treated] Rif^r CFU per 10⁸ survivors) or WFP340 (6.4 ± 3.2 [untreated] versus 3.5 ± 2.3 [MMC treated] Rif^r CFU per 10⁸ survivors).

Reaction of nitrofurazone (NFZ) or 4-nitroquinoline 1-oxide (4-NQO) with DNA results primarily in N²-dG adducts (65). The *dinB*-encoded Pol IV of *E. coli* was recently reported to play an important role in protecting *E. coli* from the lethal effects of NFZ and 4-NQO by catalyzing largely accurate TLS over NFZ- and 4-NQO-induced N²-dG adducts (28). We therefore examined whether *dinB*_{Pa} played a similar role in protecting *P. aeruginosa* against the lethal effects of NFZ and 4-NQO by comparing the growth of PAO1 (*dinB*⁺) and WFP340 (Δ *dinB*::*aacC1*) on LB plates supplemented with different concentrations of NFZ or 4-NQO. Consistent with the findings of Jarosz et al. (28), the inactivation of *dinB* sensitized *E. coli* to the potentially lethal effects of NFZ (Fig. 5A, compare growth of the *dinB*⁺ and Δ *dinB* strains at the 10⁻⁴ and 10⁻⁵ dilutions on plates containing 0 or 10 μ M NFZ); however, in contrast to findings of Jarosz et al. (28), the inactivation of *dinB* did not appear to significantly sensitize *E. coli* to 4-NQO (Fig. 5C, compare growth of the *dinB*⁺ and Δ *dinB* strains at the 10⁻² and 10⁻³ dilutions on plates containing 0 or 40 μ M 4-NQO). The inactivation of *dinB* slightly sensitized *P. aeruginosa* to NFZ, although considerably higher concentrations of the NFZ were required in order to observe killing (Fig. 5A, compare growth of *dinB*⁺ and *dinB* strains at the 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions on plates containing 0, 160, and 320 μ M NFZ, respectively). In contrast to the *E. coli* Δ *dinB* control, the Δ *dinB* *P. aeruginosa* strain was significantly more sensitive to 4-NQO than was the isogenic *dinB*⁺ *P. aeruginosa* strain (Fig. 5C).

We next asked whether NFZ or 4-NQO treatment increased the frequency of Rif^r in *P. aeruginosa*. Surprisingly, unlike with most TLS, which is error prone, *dinB* of *E. coli* is able to replicate NFZ- and 4-NQO-induced lesions in a largely accurate manner and hence DinB_{Ec}-mediated TLS over NFZ-induced lesions fails to result in an increased frequency of Rif^r in

FIG. 5. Role of DinB_{Pa} in tolerating NFZ- and 4-NQO-induced DNA damage. Relative sensitivities to NFZ (A) or 4-NQO (C) of *dinB*⁺ and Δ *dinB* *E. coli* and *P. aeruginosa* strains. Cultures of *E. coli* strains RW118 (*dinB*⁺) and RM137 [Δ (*dinB-yafN*):*kan*], and of *P. aeruginosa* strains PAO1 (*dinB*⁺) and WFP334 (Δ *dinB::aacC1*) were serially diluted in 0.8% NaCl and spotted on LB plates containing the indicated concentration of NFZ or 4-NQO. Plates were incubated overnight at 37°C prior to photographing. NFZ- and 4-NQO-induced mutation frequencies were measured by inoculating 5 ml of LB containing either *N,N*-dimethylformamide (DMF), 160 μ M NFZ, or 320 μ M NFZ (B) or either 160 μ M or 320 μ M 4-NQO (D) (each made freshly in DMF [NFZ] or ethanol [4-NQO]) with 0.1 ml of *P. aeruginosa* strains PAO1 or WFP334 grown to an OD₅₉₅ of ~0.5. Following growth for ~16 h at 37°C, cells were washed twice with 0.8% NaCl and appropriate dilutions were spread onto LB or LB-Rif agar plates. Mutation frequencies were calculated by dividing the number of Rif^r CFU by the total number of viable cells for each strain. Control experiments indicated that the presence of DMF alone had no effect on the frequency of Rif^r for either PAO1 or WFP334. Mutation frequencies are expressed relative to those observed for strain PAO1 grown in the presence of DMF alone (2.9 $\pm$ 1.9 Rif^r CFU per 10⁸ CFU for the NFZ experiment), or in LB (4.9 $\pm$ 2.2 Rif^r CFU per 10⁸ CFU for the 4-NQO experiment), which were each set equal to 1.0. Results shown represent the averages of at least three independent experiments, each performed in duplicate. Error bars represent standard deviations. Differences in the frequencies of Rif^r between strains PAO1 and WFP334 grown in LB or in the presence of DMF alone (*P* value $\geq$ 0.100), relative to that grown in the presence of 4-NQO or NFZ (*P* values $\geq$ 0.220, respectively, relative to the DMF-alone or LB-only controls), were not statistically significant at the 95% confidence level based on the Student *t* test. *Ec dinB*⁺, *dinB*_{Ec}⁺; *Pa dinB*⁺, *dinB*_{Pa}⁺.

E. coli (28). In order to determine whether NFZ-induced DNA lesions are tolerated in an accurate or an error-prone way by DinB_{Pa}, we compared the frequencies of Rif^r of *P. aeruginosa* PAO1 (*dinB*⁺) and WFP334 (Δ *dinB::aacC1*) cultures grown overnight in the presence of various concentrations of NFZ or 4-NQO. Although the strain bearing the Δ *dinB::aacC1* allele grown in the presence of 160 or 320 μ M NFZ consistently displayed a higher frequency of Rif^r of about two- to threefold relative to the same strain grown in the absence of NFZ or the isogenic *dinB*⁺ strain PAO1 (Fig. 5B), this effect was not statistically significant (see the legend for Fig. 5). Similar results were observed with 4-NQO (Fig. 5D). Thus, we conclude that DinB_{Pa} protects *P. aeruginosa* against the potentially lethal effects of NFZ and 4-NQO by mediating largely accurate TLS over N²-dG adducts, as was reported for DinB_{Ec} (28).

Both *polA* (DNA polymerase I) and *polC* (the second DNA polymerase III enzyme), but not *dinB*, are required for UV-induced mutagenesis in *P. aeruginosa*. There are conflicting reports in the literature as to whether *P. aeruginosa* displays UV-induced mutagenesis (36, 44). In order to determine whether the prototrophic *P. aeruginosa* strain PAO1 displays UV-induced mutability, frequencies for spontaneous and UV-induced Rif^r were measured. As summarized in Table 5, *P. aeruginosa* displayed a modest UV-induced mutator phenotype based on the approximately twofold increase in the frequency

of Rif^r of *P. aeruginosa* strain PAO1 following UV irradiation (*P* value of 0.041). Since *P. aeruginosa* lacks an *E. coli umuC* (Pol V) ortholog, we asked whether DinB_{Pa} played a role in UV-induced mutagenesis. Our finding that the frequencies of UV-induced Rif^r were comparable in both the *dinB*⁺ (PAO1)

TABLE 5. Spontaneous and UV-induced mutation frequencies in *P. aeruginosa dinB*⁺ and Δ *dinB* strains

<i>P. aeruginosa</i> strain ^a	<i>dinB</i> allele	Rif ^r CFU/10 ⁸ CFU $\pm$ SEM ^b		Ratio of UV-induced/spontaneous (<i>P</i> value) ^c
		Spontaneous	UV-induced	
PAO1	<i>dinB</i> ⁺	2.30 $\pm$ 0.80	4.80 $\pm$ 2.50	2.1 (0.041)
WFP334	Δ <i>dinB::aacC1</i>	3.50 $\pm$ 1.40	9.80 $\pm$ 5.10	2.8 (0.015)

^a *P. aeruginosa* strains are described in Table 1.
^b UV-induced mutation frequencies were determined as described previously (36, 45). Spontaneous mutation frequencies were determined in the same manner using mock-irradiated cultures. Values presented for spontaneous and UV-induced mutation frequencies represent the averages from at least six independent experiments $\pm$ the standard error of the means. Differences between respective spontaneous and UV-induced mutation frequencies of PAO1 and WFP334 were not statistically different at the 95% confidence level based on the Student *t* test (*P* values of 0.100 and 0.057, respectively). Median (*m*) and range (*r*) values for spontaneous mutation frequencies are as follows: for PAO1, *m* = 2.5 and *r* = 1.0 to 3.0, and for WFP334, *m* = 4.0 and *r* = 1.0 to 4.0.
^c Ratios represent the increase (*n*-fold) in mutation frequency following UV irradiation (relative to the spontaneous mutation frequency).

FIG. 6. UV-induced mutagenesis in *P. aeruginosa*. UV-induced mutagenesis was performed as described previously (36, 44) using the indicated isogenic *P. aeruginosa* strains expressing all five known *P. aeruginosa* DNA polymerases (MPAO1), or bearing a transposon insertion in the gene encoding either the DNA polymerase I (*polA*; MPA50455), DNA polymerase II (*polB*; MPA17464) or the second DNA polymerase III enzyme (*polC*; MPA34086). Results shown represent the averages of at least three independent experiments, each performed in duplicate. Error bars represent standard deviations. Based on the Student *t* test, differences between strains MPAO1 and MPA17464 are not statistically significant at the 95% confidence level (*P* value of 0.79), while those between strains MPAO1 and MPA50455 (*P* value of 0.03) as well as MPAO1 and MPA34086 (*P* value of 0.002) are statistically significant.

and Δ *dinB::aacC1* strains (WFA334) indicated that DinB_{Pa} does not play an essential role in UV-induced mutagenesis in *P. aeruginosa* (Table 5). Moreover, our finding that the frequency of spontaneous Rif^r in the WFA334 strain was essentially indistinguishable from that of PAO1 suggests that DinB_{Pa} does not contribute significantly to spontaneous mutagenesis in *P. aeruginosa* under the conditions examined in this study (Table 5), consistent with what has been reported for *dinB* in *P. putida* (59).

Inspection of the PAO1 genome indicates that it encodes at least five distinct DNA polymerases. These include, in addition to the *dinB*-encoded Pol IV (PA0923), one Pol I (PA5493), one Pol II (PA1886), and two Pol IIIs (PA3640 and PA0669). Although Pol I has been reported to participate in mutagenesis in *Bacillus subtilis* (17), it does not play a role in mutagenesis in *E. coli*. In contrast, *E. coli* Pol II does participate in TLS under certain conditions (2, 4, 31) and the *P. aeruginosa* Pol II may likewise contribute to mutagenesis in *P. aeruginosa*. PA3640 is more closely related to the catalytic subunit of the *E. coli* replicase that is encoded by *dnaE*. This gene is presumably essential for the viability of *P. aeruginosa* (27), suggesting that PA3640 encodes the *P. aeruginosa* replicase. In contrast to PA3640, PA0669 is dispensable for viability in *P. aeruginosa* (27). Interestingly, both *B. subtilis* (16) and *Caulobacter crescentus* (23) also possess two Pol IIIs, encoded by the *dnaE* and *polC* genes and by the *dnaE1* and *dnaE2* genes, respectively. *B. subtilis* *dnaE* (35) and *C. crescentus* (23) *dnaE2* are each DNA damage inducible and appear to be responsible for most damage-induced mutagenesis. We therefore used a collection of isogenic strains that were made available through the University of Washington Genome Center (27) to determine whether DNA Pol I (*polA* or PA5493), DNA Pol II (*polB* or PA1886), and/or the dispensable DNA Pol III (*polC* or PA0669) were involved in UV-induced mutagenesis. As shown in Fig. 6, UV-

induced mutagenesis in *P. aeruginosa* was dependent upon both DNA Pol I (*polA*) and the second DNA Pol III (*polC*).

DISCUSSION

As part of an effort to understand the mechanisms of mutagenesis in *P. aeruginosa*, we cloned the *P. aeruginosa* *dinB* gene and characterized the recombinant DinB_{Pa} protein using a combination of genetic and biochemical approaches. Results discussed above indicate that DinB_{Pa} possesses an intrinsic DNA polymerase activity in vitro without the requirement for additional partner proteins: DinB_{Pa} was distributive and lacked 3'-to-5' exonuclease proofreading activity (Fig. 1). Importantly, DinB_{Pa} was error prone when expressed from a plasmid in *E. coli*, enhancing both C→A transversion and -1 frameshift mutations (Table 3). Interestingly, DinB_{Ec} was previously reported to prefer these same mutations (33), although it was proficient at a wider spectrum of mutations, including transitions, transversions, and frameshift mutations that were not conferred by ectopic expression of DinB_{Pa} in this study (Table 3). As with *E. coli*, ectopic expression of DinB_{Pa} conferred a mutator phenotype in *P. aeruginosa* (Table 4). In contrast, the inactivation of *lexA*_{Pa} failed to affect the spontaneous mutation frequency of PAO1 as measured by Rif^r (Table 4). Our finding that *dinB* protected *P. aeruginosa* against the lethal effects of NFZ and 4-NQO without significantly affecting the frequency of induced mutagenesis (as measured by Rif^r) suggests that DinB_{Pa} promotes largely accurate TLS over N²-dG adducts (Fig. 5) as was recently reported for DinB_{Ec} (28).

In addition to our finding that DinB_{Ec} appeared more promiscuous than did DinB_{Pa} with respect to the types of mutations that it could confer, we also observed a striking difference in the behavior of equivalent mutant forms of DinB_{Ec} and DinB_{Pa}. Based on the high degree of identity (49%)/similarity (63%) between these two proteins, we hypothesized that amino acid substitutions previously described as impairing the catalytic DNA polymerase activity of DinB_{Ec} would likewise impair the activity of DinB_{Pa}. Substituting either D8, R49, or D103 with alanine was reported to severely impair DinB_{Ec}-mediated Lac⁺ reversion in vivo as well as catalytic activity of the purified protein in an in vitro primer extension assay, without noticeably affecting the stability of the recombinant protein (60). In striking contrast to these findings with DinB_{Ec}, the DinB(R49A)_{Pa} mutant protein retained partial catalytic activity as measured both in vitro using a primer extension assay (Fig. 1) as well as in vivo using a *lacZ*→Lac⁺ reversion assay (Table 3). Based on the crystal structure of the *Sulfolobus solfataricus* Dpo4 *dinB* ortholog (37), residue R49 is located in the presumed DNA binding region and/or interacts with the incoming dNTP (37). Our finding that the R49A mutant DinB_{Pa} protein retained partial activity, while the R49A mutant DinB_{Ec} protein was reported to lack detectable activity (60), suggests that this residue (R49) may play different roles in these two enzymes. Moreover, our finding that the D103A DinB_{Pa} protein was poorly stable when expressed in *E. coli* (Fig. 2) similarly contrasts with previous reports describing the DinB(D103A)_{Ec} mutant (60). Taken together, these findings suggest that residues R49 and D103 contribute differently to catalysis/folding in these two enzymes, perhaps due to subtle

differences between the structures of their respective active sites.

In an attempt to understand the regulation of *DinB_{Pa}*, we generated a Δ *lexA* mutant and compared *DinB_{Pa}* levels with those of the wild type of *P. aeruginosa*. There were higher levels of *DinB_{Pa}* in the Δ *lexA* mutant, indicating a role for LexA in repressing *DinB_{Pa}* expression (Fig. 3). This was validated by a real time RT-PCR assay in which we observed a fivefold increase in *dinB* mRNA levels in WFP340 compared with that in the wild-type *P. aeruginosa* strain. Moreover, experiments with MMC indicate that the expression of *dinB_{Pa}* is elevated in response to MMC-mediated DNA damage (Fig. 3). These results suggest that *DinB_{Pa}* is under the control of an SOS-like system which has not been well studied in *P. aeruginosa*. To directly test this possibility, recombinant LexA_{Pa} was generated and analyzed for binding to *dinB* regulatory sequences (Fig. 4). The fragment used in these binding assays contained a putative LexA box. It should be noted that, during the course of purification, we consistently recovered two distinct LexA_{Pa} species. LexA_{Ec} undergoes RecA-mediated autocatalytic cleavage during the SOS response (38). Therefore, it is likely that these two species correspond to the cleaved and uncleaved forms of LexA_{Pa}. This likelihood is supported by the molecular masses of the two proteins as well as by immunoblot analyses with anti-His antiserum (data not shown). We determined that purified LexA_{Ec} will not bind upstream of *dinB_{Pa}* at concentrations necessary to bind *E. coli umuDC* DNA (Fig. 4A and B). The binding of LexA_{Pa} to *dinB* requires significantly higher concentrations of protein than that required for LexA_{Ec} to bind its target DNAs. There are several possible explanations for these differences. First, it is possible that LexA_{Pa} exhibits overall weaker binding affinity for target sequences or that the *dinB_{Pa}* LexA box is of low affinity compared with those of other LexA-repressed genes. It is also possible that the two species of LexA in our preparations antagonize the binding activity, or that some DNA binding activity is lost during purification. Nevertheless, the Western blot and real-time RT-PCR data (Fig. 3), taken together with the LexA binding studies (Fig. 4), lead us to conclude that *DinB_{Pa}* is most likely under the direct control of LexA_{Pa}. The closely related *Pseudomonas putida* also encodes a *DinB* protein that is directly regulated by LexA (1). Our conclusion that *dinB_{Pa}* is regulated as part of an SOS-like response is further supported by the finding that the transcription of *dinB_{Pa}* was induced following exposure to hydrogen peroxide (47). On the other hand, there are conflicting reports in the literature as to whether ciprofloxacin (a fluoroquinolone), which is reported to induce the SOS response in *Haemophilus influenzae* and *P. aeruginosa* (11), induces *dinB_{Pa}* transcription: two independent microarray analyses failed to observe an effect of ciprofloxacin on *dinB_{Pa}* transcript levels (6, 7), while a third microarray analysis reported that ciprofloxacin induced *dinB_{Pa}* transcription in a LexA-independent fashion (11). Further work is necessary in order to better understand the effects that these antibiotics have on LexA-dependent regulation of *dinB* transcription.

Various mutations have been described that confer patho-adaptive value upon *P. aeruginosa*; the *mucA* gene is perhaps the best-studied locus in this regard. Although the mechanism(s) by which *mucA* acquires inactivating mutations is currently unknown, nucleotide sequence analysis of *mucA* alleles

impaired for the regulation of AlgT have been previously described. Interestingly, a remarkably large fraction of these *mucA* alleles were reported to contain a -1 frameshift mutation located within a homopolymeric run of 5 deoxyguanylic acid residues (known as the *mucA22* allele) (40). Thus, it is tempting to speculate that replication errors, such as those introduced by *DinB_{Pa}*, might underlie the mechanistic basis for the initial mutagenic events leading to *mucA* inactivation. Our finding that *DinB_{Pa}* efficiently promotes -1 frameshift mutations within homopolymeric runs of deoxyguanylic or deoxyadenylic acid residues (Table 3) suggests that this enzyme may play an important role in *mucA* inactivation. The finding that overexpressed levels of *DinB_{Pa}* resulted in a modest mutator phenotype is consistent with this model. Our results suggest that error-prone replication by *DinB_{Pa}* and/or *P. aeruginosa* Pols I and/or Pol III might also contribute to the hypermutable phenotype that has been described for some clinical isolates of *P. aeruginosa* recovered from individuals suffering from chronic infections. The finding that hypermutability in clinical *P. aeruginosa* isolates was largely the result of an MMR defect (46) is consistent with the idea that error-prone replication contributes to *mucA* mutagenesis. Thus, we suggest that, in the absence of MMR, replication errors introduced by Pols I, III, and IV would persist, leading to elevated levels of mutagenesis. Further genetic and biochemical analysis of *DinB_{Pa}* as well as the other *P. aeruginosa* Pols will address this possibility. Furthermore, in *C. crescentus*, two hypothetical genes, named *imuA* and *imuB* and found in the same operon as the *dnaE2*, appear to work in conjunction with *dnaE2* to promote UV- and MMC-induced mutagenesis (23). An inspection of the PAO1 genome indicates that it carries an *imuB* ortholog (PA0670). Further work is necessary in order to determine whether *imuB_{Pa}* contributes to induced mutagenesis in *P. aeruginosa*.

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