Epistatic Roles for *Pseudomonas aeruginosa* MutS and DinB (DNA Pol IV) in Coping with Reactive Oxygen Species-Induced DNA Damage

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**Abstract**

*Pseudomonas aeruginosa* is especially adept at colonizing the airways of individuals afflicted with the autosomal recessive disease cystic fibrosis (CF). CF patients suffer from chronic airway inflammation, which contributes to lung deterioration. Once established in the airways, *P. aeruginosa* continuously adapts to the changing environment, in part through acquisition of beneficial mutations via a process termed pathoadaptation. MutS and DinB are proposed to play opposing roles in *P. aeruginosa* pathoadaptation: MutS acts in replication-coupled mismatch repair, which acts to limit spontaneous mutations; in contrast, DinB (DNA polymerase IV) catalyzes error-prone bypass of DNA lesions, contributing to mutations. As part of an ongoing effort to understand mechanisms underlying *P. aeruginosa* pathoadaptation, we characterized hydrogen peroxide (*H₂O₂*)-induced phenotypes of isogenic *P. aeruginosa* strains bearing different combinations of *mutS* and *dinB* alleles. Our results demonstrate an unexpected epistatic relationship between *mutS* and *dinB* with respect to *H₂O₂*-induced cell killing involving error-prone repair and/or tolerance of oxidized DNA lesions. In striking contrast to these error-prone roles, both MutS and DinB played largely accurate roles in coping with DNA lesions induced by ultraviolet light, mitomycin C, or 4-nitroquinilone 1-oxide. Models discussing roles for MutS and DinB functionality in DNA damage-induced mutagenesis, particularly during CF airway colonization and subsequent *P. aeruginosa* pathoadaptation are discussed.

**Introduction**

Despite the fact that most organisms are equipped with numerous DNA repair functions, DNA lesions often evade repair. If left unchecked, these lesions can block ongoing replication, leading to mutations, genome rearrangements, and even cell death [1]. One evolutionarily conserved mechanism by which bacteria tolerate replication blocking DNA lesions involves their direct bypass via a process termed translesion DNA synthesis (TLS) [1,2]. Most if not all organisms possess multiple DNA polymerases (Pol) capable of catalyzing TLS, several of which belong to the Y-family [1,3]. In general, members of this family of Pol possess a preformed and open catalytic active site compared to well studied high fidelity replicative Pol, are distributive, and lack intrinsic exonuclease proofreading activity (reviewed in [1,4]). Taken together, these features confer upon Y-family Pol a reduced fidelity relative to most well studied replicative enzymes. This reduced fidelity is vital to their ability to catalyze TLS, and, together with the miscoding or non-coding nature of many lesions, explains why TLS can be error-prone, contributing to mutations.

The Y-family of Pols is comprised of four main subgroups, or branches (reviewed in [3]). The bacterial DinB (Pol IV; hereafter referred to as DinB)/eukaryotic Pol κ branch is the most evolutionarily conserved [3,5], suggesting that its members play one or more vitally important roles with respect to DNA repair/damage tolerance. Although it is unclear whether the members of the DinB branch act in one or more conserved role, several distinct activities have been described for representative members. For example, both *E. coli* DinB and mammalian Pol κ are capable of catalyzing accurate bypass of N₂-dG–furfuryl adducts [6,7], as well as model N₂-dG–N₂-dG interstrand DNA cross-links [8,9]. In addition, *E. coli* DinB plays an active role in contributing to mutations under conditions of limiting carbon source via an error-prone dsDNA break repair pathway [10], and may play a role in error-free bypass of cytotoxic alkylating DNA lesions [11]. Although *E. coli* DinB cannot catalyze bypass of UV photoproducts [12], the *Sulfolobus solfataricus* DinB ortholog, P2 Pol IV (Dpo4)

![Image](Image.png)
can bypass a model cis-syn thymine cyclobutane dimer in vitro [13]. Human Pol \( \kappa \) is unable to bypass a model thymine dimer in vitro; however, it can extend a primer bearing a 3'-dG located opposite the 3'-dT of a model thymine cyclobutane dimer in vitro [14]. Moreover, Pol \( \kappa \) catalyzes error-prone bypass of 8-oxo-7,8-dihydro-2'-deoxyguanosine 3'-monophosphate (8-oxo-dG), and largely accurate bypass of 5,6-dihydro-5,6-dihydroxythymidine (thymine glycol) in vitro [15,16,17]. Finally, both E. coli DinB and human Pol \( \kappa \) incorporate 2-hydroxy-dATP opposite template-dG or -dT, as well as 8-oxo-dG opposite template-dA [18,19]. Taken together, these findings illustrate the difficulty in predicting a priori the ability of a particular DinB/Pol \( \kappa \) enzyme to tolerate a specific lesion, and/or the fidelity with which a particular DinB/Pol \( \kappa \) enzyme will behave.

We recently initiated a study of the Pseudomonas aeruginosa DinB protein as part of a larger effort aimed at understanding mechanisms contributing to mutagenesis and adaptation [20]. P. aeruginosa is a human opportunistic pathogen that is commonly associated with a variety of human diseases, particularly chronic respiratory infections of cystic fibrosis (CF) patients (reviewed in [21,22]). Following airway colonization, P. aeruginosa acquires mutations that confer an adaptive advantage, enabling the pathogen to persist within CF airways for years to decades, ultimately leading to the death of the patient [23,24,25,26,27,28,29,30]. The process by which P. aeruginosa acquires adaptive mutations is referred to as ‘pathoadaptation.’

Mutational inactivation of the P. aeruginosa mucA gene results in a mucoid phenotype, and is one of the best-studied examples of an adaptive mutation directly correlated with persistent infections and poor clinical prognosis (reviewed in [31]). Based on two separate studies [32,33], more than 80% of the mucoid P. aeruginosa strains isolated from individuals afflicted with CF were found to contain a mutation within mucA, suggesting that mutations inactivating this locus confer an advantage in airway pathogenesis. The mucA gene encodes an anti-sigma factor (MucA protein) that negatively regulates the actions of the alternative sigma factor, AlgT (or AlgU) [reviewed in [23,31]]. Inactivation of mucA leads to loss of MucA-mediated antagonism of AlgT, which in turn activates transcription of genes required for alginate production. A majority of mucA alleles impaired for regulation of AlgT contain a -1 frameshift mutation within a single homopolymeric run of 5 consecutive dG residues, referred to as the mucA22 allele [32]. Our finding that P. aeruginosa DinB favored -1 frameshift mutations within poly-dA and poly-dG runs over GC→TA transversions nearly 3-to-1 led us to suggest that this Pol may contribute to mucA inactivation during airway infection [20]. Consistent with this hypothesis, DinB contributes to mutations in mucA that promote alginate production under laboratory conditions [34]. This same study revealed that mutational inactivation of MutS, which together with MutL and UvrD enables mismatch repair (MMR), served to significantly increase the frequency of mucA mutations.

MMR is a replication-coupled repair function that acts to correct replication errors, thereby limiting the frequency of spontaneous mutations. MMR function relies at a minimum on MutS, MutL, and UvrD. Based on seminal work in E. coli, MutS acts to recognize DNA mismatches [reviewed in [35]]. After binding the mismatch, MutS recruits MutL. In some organisms, MutL possesses a nuclease activity that cleaves the daughter strand containing the replication error [36]. P. aeruginosa MutL has not yet been demonstrated to possess nuclease activity. MutL also acts to recruit UvrD, a DNA helicase (helicase II) that loads at the nick site to unwind the duplex DNA, leaving a single strand (ss) DNA gap. This ssDNA gap is subsequently filled in by action of the replicative DNA polymerase, and the resulting nick sealed by DNA ligase. Loss-of-function mutations in either mutS, mutL, or uvrD abolish MMR, leading to significantly elevated spontaneous mutation frequencies, due in large part to an inability to correct replication errors [35]. More recently, roles for MutS, MutL, and UvrD in addition to MMR have been suggested. For example, MutS may participate in base excision repair. MutL was recently determined to interact with a variety of proteins that participate in functions other than MMR, and UvrD participates in nucleotide excision repair, and also plays an antagonistic role in recombination by dismantling RecA/ssDNA nucleoprotein filaments [37,38,39,40,41]. Taken together, these findings suggest that DNA lesions in addition to mismatched base pairs might contribute to the elevated mutation rates observed for MMR-deficient strains. Finally, of significance to work discussed in this report, a significant fraction of CF patients harbor a population of P. aeruginosa that displays an elevated spontaneous mutation frequency (e.g., hypermutable phenotype) due to loss of MMR function, most frequently resulting from mutational inactivation of mutS [24,25,28,42].

Despite the fact that a large fraction of CF patients are colonized by mutS-deficient P. aeruginosa strains [24,25,28,42], there is a paucity of information regarding the biology of these strains. Based on a murine airway infection model, inactivation of mutS renders P. aeruginosa less virulent [28,43]. P. aeruginosa is exposed to high levels of reactive oxygen species (ROS) while colonizing CF airways, especially in the early “aerobic” phases (reviewed in [21,22]). As a result, P. aeruginosa experiences significant levels of DNA damage. Inasmuch as MutS acts to prevent mutations, we hypothesized that MutS function might contribute to accurate repair of ROS-damaged DNA, and that in the absence of MutS, P. aeruginosa would be impaired for coping with these lesions, possibly explaining the reduced virulence of this strain in a murine model [28,43]. Moreover, since oxidized DNA lesions can be tolerated by TLS, we further hypothesized an important role for DinB in protecting P. aeruginosa against ROS-induced DNA damage. Finally, since error-prone repair or TLS of oxidized DNA lesions will contribute to mutations, we hypothesized that MutS and DinB play opposing roles in P. aeruginosa pathoadaptation. While testing these hypotheses using isogenic mutS- and/or dinB-deficient strains, we unraveled an unexpected epistatic relationship between mutS and dinB functionality with respect to H₂O₂-induced cell killing. Our discovery that both dinB and mutS functionality contributed to H₂O₂-induced mutagenesis suggests that H₂O₂ sensitivity of the mutS and dinB strains was the direct result of their impaired ability to cope with ROS-induced DNA damage. In striking contrast to these error-prone roles, both MutS and DinB played accurate roles in tolerating and/or repairing DNA lesions induced by ultraviolet light (UV), mitomycin C (MMC), or 4-nitroquinilone 1-oxide (4-NQO) exposure. Models discussing roles for MutS and DinB functionality in DNA lesion repair and/or tolerance, as well as DNA damage-induced mutagenesis, particularly during CF airway colonization and subsequent P. aeruginosa pathoadaptation are discussed.

Results

Contributions of P. aeruginosa DinB and MutS to spontaneous mutagenesis

As part of an ongoing effort to understand mechanisms contributing to P. aeruginosa pathoadaptation, we constructed a set of isogenic PAO1-derived strains bearing different combinations of wild-type and mutant mutS and dinB alleles. Prior to determining roles for dinB and/or mutS function(s) in DNA damage-induced mutagenesis, we measured their respective
contributions to spontaneous mutagenesis. Inactivation of *mutS* (*mutS*: *isphaA*) conferred a ~650-fold increase in the frequency of spontaneous Rif<sup>R</sup> compared to the isogenic *mutS* strain (Table 1), consistent with its well-established role in replication-coupled MMR [35]. Hypermutability of the *mutS* strain was fully complemented by plasmid pmutS (Table 1), which expresses the *P. aeruginosa* MutS protein from its native promoter. In contrast to *mutS*, deletion of *dinB* (*dinB*: *aac(C)I*) failed to significantly influence the frequency of spontaneous Rif<sup>R</sup>, irrespective of *mutS* activity (Table 1). Based on nucleotide sequence analysis of representative spontaneously arising Rif<sup>R</sup> clones for each of the four strains, mutations resulted exclusively from spontaneous base substitutions (Table 2), as expected given the essential role of the *ispB* gene product (β subunit of RNA Pol) [44]. The overwhelming majority of observed mutations were TA→CG transitions, irrespective of *mutS* and *dinB* functionality (see Fig. 1A & Table 2). Importantly, GC→TA transitions, which are characteristic of DinB [20], were not observed. Taken together, these results indicate that DinB function does not contribute to spontaneous base substitutions in *P. aeruginosa*. In addition, they confirm a pivotal role for MutS in limiting spontaneous mutations.

### DinB and MutS act epistatically to protect *P. aeruginosa* against H<sub>2</sub>O<sub>2</sub>-mediated killing

The *dinB* and *mutS* genes are among the numerous *P. aeruginosa* genes whose transcription is induced following exposure to H<sub>2</sub>O<sub>2</sub> [45,46], suggesting roles for DinB and MutS in coping with ROS-induced DNA lesions. To test this hypothesis, we asked whether loss of *dinB* and/or *mutS* function(s) enhanced sensitivity of *P. aeruginosa* to H<sub>2</sub>O<sub>2</sub>. As summarized in Fig. 2A, wild-type *P. aeruginosa* (MPAO1) was relatively insensitive to killing by H<sub>2</sub>O<sub>2</sub> over the concentration range examined. In contrast to MPAO1, the *mutS*-deficient strain (MPA32417) displayed a pronounced hypersensitivity to all H<sub>2</sub>O<sub>2</sub> concentrations examined (Fig. 2A), suggesting an important role for MutS in sensing and/or repairing oxidized DNA lesions. H<sub>2</sub>O<sub>2</sub> hypersensitivity was statistically significant (*p*<0.05, based on two-way ANOVA with Bonferroni post-test), and was fully complemented by the MutS-expressing plasmid (pmutS). The *dinB*-deficient strain (WFPA334) also displayed increased sensitivity to H<sub>2</sub>O<sub>2</sub>, although this sensitivity was largely restricted to higher levels of H<sub>2</sub>O<sub>2</sub> (Fig. 2A), suggesting an important role for DinB in tolerating H<sub>2</sub>O<sub>2</sub>-induced DNA lesions that persist in the DNA, rather than their repair. Sensitivity of the *dinB* strain was statistically significant at concentrations ≥200 mM H<sub>2</sub>O<sub>2</sub> (*p*<0.05), and was fully complemented by the DinB-expressing plasmid, pAR101. Finally, the *mutS dinB* double mutant strain (UBPA100) displayed a level of H<sub>2</sub>O<sub>2</sub> sensitivity that was comparable to the *mutS*-deficient *dinB* strain (Fig. 2A), indicating an epistatic relationship between *dinB* and *mutS* function(s) with respect to H<sub>2</sub>O<sub>2</sub>-mediated killing. As a control, we measured levels of catalase activity in cell-free extracts prepared from each strain. As summarized in Fig. 2B, each strain harbored comparable levels of catalase activity (*p*>0.05, based on one-way ANOVA with Dunnett’s post-test), indicating that H<sub>2</sub>O<sub>2</sub> sensitivity of the *mutS* and *dinB* strains was not the result of reduced catalase levels. These results, taken together with the known roles for DinB and MutS discussed above, support a model in which *P. aeruginosa* MutS and DinB act in a common pathway focused on coping with oxidized DNA damage.

### Both DinB and MutS contribute to H<sub>2</sub>O<sub>2</sub>-induced mutagenesis in *P. aeruginosa*

We hypothesized that H<sub>2</sub>O<sub>2</sub>-sensitivity of *dinB* and/or *mutS* strains was the result of their respective abilities to effectively cope with oxidized DNA lesions. Given that DinB is a TLS Pol, we hypothesized that it might catalyze error-prone bypass of oxidized DNA lesions, resulting in H<sub>2</sub>O<sub>2</sub>-induced mutations. In this case, inactivation of DinB would reduce the frequency of H<sub>2</sub>O<sub>2</sub>-induced mutagenesis. In addition, we hypothesized that independently of DinB, MutS might recognize oxidized DNA lesions, and either directly catalyze their repair, or shuttle them into the appropriate accurate DNA repair pathway. In this case, loss of MutS function would result in accumulation of H<sub>2</sub>O<sub>2</sub>-induced lesions, possibly necessitating their tolerance via DinB-mediated TLS. As a test of these hypotheses, we measured H<sub>2</sub>O<sub>2</sub>-induced mutation frequencies for our isogenic *mutS* and/or *dinB* strains. As summarized in Fig. 3A, mutation frequency of the wild-type *P. aeruginosa* strain (MPAO1) was increased ~10-fold following exposure to H<sub>2</sub>O<sub>2</sub> (20.7±1.5×10<sup>−8</sup>) compared to the mock treated control (2.2±1.5×10<sup>−8</sup>). Since we propose that DinB activity contributes to this mutator phenotype, we tested if the loss of DinB function impaired H<sub>2</sub>O<sub>2</sub>-induced mutagenesis. Consistent with our hypothesis, the frequency of H<sub>2</sub>O<sub>2</sub>-induced mutagenesis for the *dinB*-deficient strain (5.6±1.0×10<sup>−8</sup>) was ~4-fold lower than that observed with the wild-type strain (20.7±1.3×10<sup>−8</sup>; see Fig. 3A). Importantly, H<sub>2</sub>O<sub>2</sub>-induced mutagenesis was fully restored by the DinB-expressing plasmid, verifying a direct role for DinB.

We next asked whether MutS function influenced the frequency of H<sub>2</sub>O<sub>2</sub>-induced mutagenesis. As shown in Fig. 3B, the *mutS*-deficient *dinB* strain (MPA32417) displayed a weak
Table 2. Nucleotide sequence analysis of *rpoB* alleles recovered from spontaneous or H₂O₂-induced Rif⁺ *P. aeruginosa* strains.a

| Spontaneous *rpoB* mutations | Deduced amino acid substitution | Rif⁺ *P. aeruginosa* strains
|------------------------------|---------------------------------|-----------------------------|
| Nucleotide substitution      |                                 | MPAO1 (WT)¹ | WFPA334 (dinB)² | MPA32417 (mutS)³ | UBPA100 (mutS dinB)⁴ |
| CTG→CCG                      | L516→P                          | nd          | nd              | nd              | 1                |
| TCG→CCG                      | S517→P                          | 1           | nd              | nd              | 1                |
| CAG→CGG                      | Q518→R                          | 4           | nd              | nd              | 4                |
| GAC→AAC                      | D521→N                          | 1           | nd              | nd              | 1                |
| GAC→GGG                      | D521→G                          | 3           | 14              | 15              | 12               |
| GAC→GTC                      | D521→V                          | 1           | nd              | nd              | nd               |
| GAC→GCC                      | D521→A                          | nd          | 1'              | nd              | nd               |
| CAG→GAG                      | Q522→E                          | nd          | 1'              | nd              | nd               |
| AAC→GAC                      | N523→D                          | nd          | 1              | nd              | nd               |
| CAC→TAC                      | H531→Y                          | nd          | 1              | nd              | nd               |
| CAC→GGG                      | H531→R                          | nd          | 1              | 3               | 1                |
| TCC→TTC                      | S536→F                          | 1           | nd              | nd              | nd               |
| CCT→CCC                      | L538→P                          | 1           | nd              | nd              | nd               |

| H₂O₂-induced *rpoB* mutations | Deduced amino acid substitution | Rif⁺ *P. aeruginosa* strains
|------------------------------|---------------------------------|-----------------------------|
| Nucleotide substitution      |                                 | MPAO1 (WT)¹ | WFPA334 (dinB)² | MPA32417 (mutS)³ | UBPA100 (mutS dinB)⁴ |
| CTG→CCG                      | L516→P                          | nd          | nd              | nd              | 1                |
| TCG→CCG                      | S517→P                          | nd          | nd              | 1               | nd               |
| TCG→TTG                      | S517→L                          | nd          | 1              | nd              | 1                |
| CAG→CGG                      | Q518→R                          | nd          | nd              | 1               | nd               |
| GAC→AAC                      | D521→N                          | nd          | nd              | 2               | nd               |
| GAC→GGG                      | D521→G                          | 10          | 8              | 15              | 17               |
| CAC→GAC                      | H531→D                          | 3           | 6              | nd              | nd               |
| CAC→TAC                      | H531→Y                          | nd          | 2              | nd              | nd               |
| CAC→CTC                      | H531→L                          | 2           | nd              | nd              | nd               |
| CCT→CCC                      | L538→P                          | 4           | nd              | nd              | nd               |
| CCT→CTT                      | P567→L                          | 1           | nd              | nd              | nd               |

aSpontaneous and H₂O₂-induced (25 mM H₂O₂) mutations were identified as described in Materials and Methods. The region of *rpoB* encompassing amino acids 499–582 of the β subunit of RNA polymerase was PCR amplified from 18–20 independent Rif⁺ clones for each strain and subjected to automated nucleotide sequence analysis.

bEach nucleotide substitution (underlined) is shown in the context of its respective codon.

The number of times that each mutation was identified in the group of 18–20 that was sequenced is indicated; *nd*, the indicated mutation was not detected.

Seven of the 19 Rif⁺ clones examined did not contain a mutation within amino acids 499–582.

One of the 19 Rif⁺ clones examined did not contain a mutation within amino acids 499–582.

These mutations were present in the same *rpoB* allele.

Three of the 20 Rif⁺ clones examined did not contain a mutation within amino acids 499–582.

Two of the 20 Rif⁺ clones examined did not contain a mutation within amino acids 499–582.

H₂O₂-induced mutator phenotype that was only ~1.5-fold higher than its spontaneous mutation frequency (26.5±3.2×10⁻⁶ for H₂O₂-treated cultures compared to 18.2±5.9×10⁻⁶ for mock-treated controls). Inasmuch as this increase was considerably smaller than the ~10-fold increase observed for the *mutS* dinB strain (MPAO1), these results suggest a direct role for MutS in ROS-induced mutagenesis (see Discussion). Importantly, H₂O₂-induced mutagenesis in the *mutS* strain was still DinB-dependent (Fig. 3B), as we observed a more than 8-fold reduction in the H₂O₂-induced mutation frequency for the *mutS dinB* strain (3.3±1.9×10⁻⁶) compared to the *mutS*-deficient *dinB* strain (26.5±3.2×10⁻⁶). Consistent with results discussed above, this defect was fully complemented by the DinB-expressing plasmid, providing further support for a role for DinB (Fig. 3B). Remarkably, the frequency of Rif⁺ for the H₂O₂-treated *mutS dinB* strain (3.3±1.9×10⁻⁶) was more than 6-fold lower than the spontaneous mutation frequency observed for the same strain following mock treatment (21.5±4.5×10⁻⁶), resulting in an overall negative value for H₂O₂-induced mutation frequency (~18.2±4.7×10⁻⁶; Fig. 3B). This difference was reproducibly observed, and was statistically significant (*p*<0.001, based on Student’s *t*-test). Taken together, these results support a model in which DinB and MutS act synergistically to cope with oxidized DNA lesions (see Discussion).

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**P. aeruginosa DinB catalyzes bypass of 8-oxo-dG in vitro**

We interpreted our finding that dinB function contributes to H$_2$O$_2$-induced mutagenesis (Fig. 3) to suggest that DinB catalyzes TLS over one or more classes of oxidized DNA lesions. Since 8-oxo-dG is a well-established ROS-induced lesion [1], we tested whether purified DinB protein could catalyze bypass of template–8-oxo-dG in vitro. For these experiments, we utilized a synthetic oligonucleotide substrate comprised of 20-mer template bearing either dG, or 8-oxo-dG positioned immediately downstream of the 3’-OH of an annealed complimentary 13-mer oligo (see legend to Table 3 for oligo sequences). Under single turnover conditions (e.g., 200 nM DinB and 100 nM DNA), DinB specifically incorporated dCTP opposite template-dG: we did not detect incorporation of dATP, dGTP, or dTTP (Table 3). In contrast to template-dG, DinB incorporated both dCTP and dATP opposite template–8-oxo-dG: incorporation of dGTP or dTTP was not detected (Table 3). The 2-fold preference for incorporation of dATP compared to dGTP arises primarily through an increase in binding affinity for dATP ($K_M = 15 \pm 5.9$ nM) relative to dCTP ($K_M = 29 \pm 7.0$ nM; see Table 3). Taken together, these findings indicate that DinB can bypass template–8-oxo-dG in either an accurate manner by inserting dCTP, or an error-prone manner by inserting dATP, contributing to GC$\rightarrow$TA transversions. Coincidently, *P. aeruginosa* DinB catalyzes GC$\rightarrow$TA transversions when expressed in *E. coli* [20].

To determine whether GC$\rightarrow$TA transversions induced by DinB-mediated bypass of 8-oxo-dG contributes to H$_2$O$_2$-induced mutagenesis in *P. aeruginosa* in vivo, we analyzed the sequence of the *rpoB* gene from representative Rif$^R$ clones (see Materials and Methods). As noted above, and summarized in Fig. 1A, the majority of spontaneous mutations resulted from TA$\rightarrow$CG transitions, irrespective of dinB and/or mutS function: 9/12 (75%) Rif$^R$ clones contained at least one TA$\rightarrow$CG transition.

![Figure 1. Summary of spontaneous and H$_2$O$_2$-induced base substitutions in *rpoB* that confer Rif$^R$. Results of *rpoB* DNA sequence analysis for spontaneous (A) and H$_2$O$_2$-induced (B) Rif$^R$ *P. aeruginosa* mutants are summarized with respect to the types of nucleotide substitution observed. Frequency refers to the occurrence of each observed base substitution mutation as a function of the total number of spontaneous or H$_2$O$_2$-induced Rif$^R$ mutants sequenced for each strain. See Table 2 for details concerning the number of Rif$^R$ clones analyzed for each strain, as well as the specific nucleotide position and substitution of each documented mutation.](doi:10.1371/journal.pone.0018824.g001)

**Figure 2. Contributions of dinB and/or mutS function to survival following exposure to ROS.** Respective H$_2$O$_2$ sensitivities (A), and catalase activities for cell free extracts (B) of isogenic *P. aeruginosa* strains MPA01 (wild-type) bearing pUCP20T, WFPA334 (ΔdinB::aacC1) bearing pUCP20T (control) or pPAR101 (dinB$^+$), MPA32417 (mutS::SphoI/hah) bearing pUCP20T (control) or pmutS (mutS$^+$), and UBPA100 (ΔdinB::aacC1 mutS::SphoI/hah) bearing pUCP20T were determined as described in Materials and Methods. H$_2$O$_2$ sensitivities represent the average of 4 independent experiments, while catalase activities represent the average of 3 independent experiments; error bars represent the standard deviation. Based on a two-way ANOVA with Bonferroni post-test, there was a significant interaction between strain and concentration of H$_2$O$_2$ in (A), and differences in H$_2$O$_2$ sensitivity of wild-type and mutS and dinB strains were statistically significant (p<0.05). Based on a one-way ANOVA with Dunnett’s post-test, differences in catalase activity in (B) were not significant (p>0.05).

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mutations in the wild-type strain resulted from TA→CG transitions, compared to 16/19 (85%) for the dinB strain, 18/18 (100%) for the mutS strain, and 19/20 (95%) for the dinB mutS double mutant. In contrast to spontaneous mutations, which at least in the wild-type strain (MPAO1) were distributed among seven distinct nucleotide positions, corresponding to five different amino acids, H2O2-treatment resulted in an obvious increase in the frequency of mutation at residue D521, identifying this position as a ‘hot spot’ for ROS-induced RifR mutations in P. aeruginosa (Table 2). Furthermore, with the exception of the dinB strain (WFPA334), which contained comparable levels of TA→CG transitions and CG→RC transversions, H2O2 treatment induced mostly TA→CG transitions (see Fig. 1B & Table 2). It is noteworthy that GC→TA transversions, characteristic of error-prone DinB-mediated bypass of 8-oxo-dG (see Table 3), were not observed. Taken together, these results suggest that P. aeruginosa DinB contributes to H2O2-induced mutagenesis by one or more mechanisms that are functionally distinct from that involving its ability to catalyze error-prone bypass of 8-oxo-dG (see Discussion).

Both DinB and MutS functionality contribute to accurate tolerance of DNA lesions induced by exposure to ultraviolet light (UV), mitomycin C (MMC), or 4-nitroquinoline 1-oxide (4-NQO) in vivo

As part of an effort to better understand the synergistic relationship between MutS and DinB in H2O2-induced mutagenesis, we asked whether these proteins played similar roles in tolerating DNA lesions induced by exposure to UV, MMC, or 4-NQO. We first determined contributions of dinB and/or mutS functionality to UV-induced mutagenesis. Based on results of previously published experiments utilizing mutS+ strains [20], Pol I and PolC are required for the vast majority of UV-induced mutagenesis: in this study, inactivation of dinB failed to impact significantly on the frequency of mutations induced by UV irradiation [20]. In order to determine whether MutS (or MMR) function masked an error-prone role for DinB in UV-induced mutagenesis, we analyzed frequencies for mutS-deficient dinB+ and D dinB strains. As summarized in Fig. 4A, the mutS-deficient strain (MPA32417) displayed a modest UV-induced mutator phenotype.

Table 3. P. aeruginosa DinB catalyzes both accurate and error-prone bypass of 8-oxo-dG in vitro.

| Incorporation of nucleoside triphosphate | Opposite template base | Kinetic parameters |  |
|----------------------------------------|------------------------|-------------------|---|
|                                       |                        | $K_{\text{cat}}$ (µM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_{\text{cat}}$ (M$^{-1}$ s$^{-1}$) |
| dCTP                                  | dG                     | 5.4±2.3           | 0.120±0.010                   | 2.2×10$^4$ |
| dCTP                                  | 8-oxo-dG               | 28±7.0            | 0.066±0.005                   | 0.2×10$^4$ |
| dATP                                  | dG                     | nd                | nd                           | na$^e$     |
| dATP                                  | 8-oxo-dG               | 15±5.0            | 0.066±0.006                   | 0.4×10$^4$ |

*Bypass activity was measured in vitro using a synthetic 13-mer oligonucleotide primer (5′-TGG CAG CCG GTC A-3′) annealed to a synthetic 20-mer template strand bearing either dG or 8-oxo-dG (3′-ACC GTC GGC CAG TAxC CCA AA-5′), where x represents either dG or 8-oxo-dG.

*Neither dTTP nor dGTP were incorporated opposite template dG at a detectable level.

*Values shown represent the average of at least 3 independent experiments, ± the standard deviation.

*Incorporation of dATP opposite template dG was not detected (nd).

*Not applicable (na).

Epistatic Roles for MutS and DinB
Epistatic Roles for MutS and DinB

that was similar in magnitude (∼2-fold increase relative to the mock-treated control) to that observed for the isogenic mutS strain [20]. These findings indicate that mutS functionality does not contribute to UV-induced mutagenesis. In contrast to the mutS strain, the mutS dinB double mutant (UBPA100) displayed a statistically significant ∼2.3-fold further increase in the frequency of UV-induced mutagenesis (Fig. 4A). This increased frequency of UV-induced mutagenesis was fully complemented by the DinB-expressing plasmid (Fig. 4A), suggesting that DinB is capable of bypassing UV-induced lesions in a relatively accurate manner in vivo, and that in its absence, one or more alternative Pols catalyze less accurate bypass of UV-induced lesions (see below). Moreover, these results suggest that MutS plays an accurate role in policing the fidelity of these alternative Pols.

We next examined roles for dinB and mutS in coping with MMC-induced $N^2$-$dG$–$N^2$-$dG$ interstrand DNA cross-links at $5'$-dc$'$-dG$'$-3’ DNA sequences [47]. We previously determined that P. aeruginosa mutS$^+$ strains failed to display an MMC-induced mutator phenotype [20]. Likewise, the mutS-deficient strain failed to display a discernable MMC-induced mutator phenotype (Fig. 4B). E. coli DinB contributes to the repair of MMC-induced $N^2$-$dG$–$N^2$-$dG$ interstrand DNA cross-links [8]. Consistent with P. aeruginosa DinB similarly contributing to repair of MMC-induced lesions, simultaneous inactivation of both mutS and dinB led to a more than 10-fold increase in the frequency of MMC-induced mutagenesis (Fig. 4B). This robust MMC-induced mutator phenotype was fully complemented by the DinB-expressing plasmid (Fig. 4B). Taken together, these results suggest that DinB contributes to accurate repair/bypass of $N^2$-$dG$–$N^2$-$dG$ interstrand DNA crosslinks, and that in the absence of DinB, another Pol catalyzes error-prone bypass. Moreover, the 10-fold increase in Rif$^R$ observed for the mutS dinB double mutant suggests that: (i) P. aeruginosa has one or more Pol(s) in addition to DinB that is capable of catalyzing bypass of MMC-induced interstrand DNA crosslinks; and (ii) MutS (or MMR) corrects errors catalyzed by the Pol(s) that is used in place of DinB.

The last agent that we examined was 4-NQO. We previously determined that dinB function protected P. aeruginosa against 4-NQO-mediated killing without significantly affecting mutation frequency, suggesting that DinB catalyzed accurate bypass of 4-NQO-induced DNA lesions [20]. As summarized in Fig. 4C, the mutS-deficient strain (MPA32417) failed to display a 4-NQO-induced mutator phenotype. In contrast, the mutS dinB double mutant (UBPA100) exhibited a ∼10-fold increase in the frequency of 4-NQO-induced mutagenesis that was fully complemented by a DinB-expressing plasmid (Fig. 4C). Taken together, these results suggest that P. aeruginosa: (i) possesses one or more Pols in addition to DinB that contributes to bypass of 4-NQO-induced DNA lesions; and (ii) MutS acts to limit errors catalyzed by the Pol(s) used in place of DinB.

Taken together, results discussed above indicate that DinB function contributes to the accurate tolerance of DNA lesions induced by UV, MMC, or 4-NQO. Importantly, in the absence of DinB, P. aeruginosa is able still able to tolerate these lesions, albeit less accurately, possibly through the use of one or more alternative TLS Pols. Importantly, mutS function contributes further to the fidelity with which these lesions are tolerated. Finally, these results also demonstrate that the mutS strain is capable of a robust DNA damage-induced mutator phenotype (Fig. 1). Thus, the inability of this strain to display a significant H$_2$O$_2$-induced mutator

**Figure 4. Contribution of dinB function to UV-, MMC-, and 4-NQO-induced mutagenesis in P. aeruginosa.** DNA damage-induced mutation frequencies following exposure of MutS-deficient dinB$^-$ and ΔdinB:aacC1 P. aeruginosa strains to 25 J/m² of 254 nm UV light delivered from a germicidal bulb (A), 1 μg/ml MMC (B), or 320 μM 4-NQO (C) were determined as described in Materials and Methods. Strains examined include MPA32417 (mutS::IsphaA/hah) bearing pUCP20T, and UBPA100 (ΔdinB:aacC1 mutS::IsphaA/hah) bearing pUCP20T (control) or pAR101 (dinB$^+$). Induced mutation frequencies represent the average of 5 independent experiments. Error bars represent the standard deviation. P-values are indicated, and were calculated using the Student’s t-test.

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Discussion

In striking contrast to their largely accurate roles in coping with lesions induced by UV, MMC, or 4-NQO, MutS and DinB activities contribute to \( \text{H}_2\text{O}_2 \)-induced mutagenesis (Fig. 3). Moreover, in the absence of both DinB and MutS, \( \text{H}_2\text{O}_2 \) exposure actually increased the fidelity of \( P. \text{aeruginosa} \) DNA replication by a factor of more than 6-fold as measured by RifR. The increase in replication fidelity without a concomitant increase in the sensitivity of \( P. \text{aeruginosa} \) to \( \text{H}_2\text{O}_2 \)-induced killing (Fig. 2) suggests the presence of one or more ROS-inducible pathways that are responsible for accurate tolerance of oxidized DNA lesions. A potential candidate is the activity of other Pols. Indeed, \( P. \text{aeruginosa} \) polC and imuB encode putative Y-family Pols ([49,50]; see http://www.pseudomonas.com). Based on results of microarray experiments, transcription of both polC and imuB is induced following exposure of \( P. \text{aeruginosa} \) to \( \text{H}_2\text{O}_2 \) [45,46]. Thus, these Pols may contribute to accurate bypass of ROS-induced lesions. Alternatively, polC and imuB function may contribute to error-prone bypass, resulting in the modest level of \( \text{H}_2\text{O}_2 \)-induced mutagenesis observed in the \( \Delta\text{dinB} \) strain (Fig. 7).

Consistent with a role for DinB in contributing to \( \text{H}_2\text{O}_2 \)-induced mutations by catalyzing error-prone bypass of oxidized DNA bases, we determined that DinB favored incorporation of dATP opposite template-8-oxo-dG over dCTP by a factor of ~2 in vitro (Table 3). However, based on nucleotide sequence analysis of the \( \text{rpoB} \) gene isolated from RifR mutants, this behavior of DinB does not appear to contribute significantly to \( \text{H}_2\text{O}_2 \)-induced mutagenesis of 

Figure 5. DinB catalyzes accurate bypass of a model cis-syn thymine cyclobutane dimer \emph{in vitro}. Cartoon representation of the 13/20T=T-mer DNA template (A). T = T represents the cis-syn thymine cyclobutane dimer. Predicted sizes for the starting primer (13-mer), as well as the different possible bypass products (14-mer & 15-mer) are indicated. Representative bypass results for T4 \( \text{exo}^- \) Pol and \( P. \text{aeruginosa} \) DinB are shown (B). Positions for the primer (13-mer), and the 14-mer and 15-mer bypass products are indicated. Quantitation of the results of a representative bypass assay obtained with both T4 \( \text{exo}^- \) Pol and DinB are shown (C). doi:10.1371/journal.pone.0018824.g005

phenotype is not an artifact of the high spontaneous mutation frequency that is characteristic of \( \text{mutS} \)-deficient strains; rather, these results provide further support for a direct role for MutS in promoting \( \text{H}_2\text{O}_2 \)-induced mutagenesis.

DinB catalyzes accurate bypass of a cis-syn thymine cyclobutane dimer \emph{in vitro}

In contrast to its error-prone role in coping with \( \text{H}_2\text{O}_2 \)-induced lesions (see Table 3), results summarized in Fig. 4 suggest that DinB catalyzes accurate bypass of DNA lesions induced by UV, MMC, or 4-NQO. In order to obtain biochemical support for this conclusion, we asked if recombinant DinB protein catalyzed accurate bypass of a model cis-syn thymine cyclobutane dimer \emph{in vitro}. As a control for these experiments, we utilized an exonuclease proofreading-deficient form of the bacteriophage T4 Pol (T4 \( \text{exo}^- \) Pol), which incorporates dATP exclusively opposite the 3'-dT of the dimer, yielding almost exclusively a 14-mer product ([19]; see Fig. 5, panels B & C). In contrast to T4 \( \text{exo}^- \) Pol, DinB catalyzed insertion of dATP opposite both thymines in the dimer, yielding a 15-mer product (~25 nM), with little to no detectable 14-mer (Fig. 5, panels B & C). This result suggests that incorporation of dATP opposite the 5’-dT of the dimer by DinB is remarkably efficient following bypass of the 3’-dT of the dimer. We therefore investigated the efficiency with which DinB extended a 14-mer primer containing a 3’-dT located opposite the 3’-dT of the dimer (see Fig. 6A). As summarized in Fig. 6 (panels B & C), DinB efficiently extended the 14-mer, preferentially incorporating dATP opposite the 5’-dT of the dimer \( (K_M = 172 \pm 39 \text{ mM}, k_m = 0.12 \pm 0.01 \text{ s}^{-1}) \). These findings, taken together with those discussed above, indicate that DinB bypasses ROS-induced lesions (see Fig. 3 and Table 3) with lower fidelity than a thymine cyclobutane dimer (see Figs. 4A, 5 & 6).
induced mutagenesis by influencing the fidelity with which oxidized lesions are repaired. For example, MutS may play an error-prone role in repairing mismatches catalyzed by DinB, particularly mismatches resulting from bypass of oxidized lesions, and/or incorporation of oxidized precursors (such as 2-hydroxy-6-azaadenine and 8-oxo-dG), due to an inability to discriminate between the lesion and the undamaged base, resulting in mutations. Alternatively, MutS might recruit DinB to sites of ROS-induced lesions, contributing to their error-prone bypass. Biochemical approaches are required to test these models.

Roles for DinB/Pol κ in catalyzing largely accurate bypass of N²-dG adducts, such as those induced by 4-NQO and MMC, have been described: E. coli DinB and mammalian Pol κ can each bypass N²-dG furfuryl adducts in vitro [6], and dinB-deficient E. coli and P. aeruginosa strains are sensitive to 4-NQO [6,20]. Furthermore, it was recently reported that both E. coli DinB [8] and human Pol κ [9,53], catalyze bypass of model N²-dG–N²-dG interstrand cross-links in vitro. Moreover, functionality of these Pols served to protect cells against MMC-induced killing, presumably via their involvement in cross-link repair. Finally, DinB and Pol κ also catalyze bypass of DNA-peptide crosslinks involving the N² position of dG in vitro [53]. Taken together, these findings indicate that DinB and Pol κ promote accurate bypass of a variety of different N²-dG adducts. Our results summarized in Fig. 4B suggest that P. aeruginosa DinB plays similar roles. In addition, our findings suggest that P. aeruginosa possesses one or more additional Pols that catalyze potentially error-prone bypass of N²-dG adducts. Our results further suggest that MutS corrects errors made by these alternate Pols, ensuring accurate bypass of N²-dG adducts in vivo (Fig. 7).

Although E. coli DinB is unable to bypass UV photoproducts [12], P. aeruginosa DinB catalyzes accurate bypass of a cis-syn thymine dimer in vivo. Cartoon representation of the 14/20 T=T-mer DNA template (A). T=T represents the cis-syn thymine dimer. Sizes for the starting primer (14-mer), as well as the bypass product (15-mer) are indicated. Bypass efficiency of the 5'-dT of the dimer by DinB as a function of time in the presence of each of the four individual dNTPs is shown (B). Catalytic efficiency with which DinB mediates bypass of the 5'-dT of the dimer in the presence of dATP (C). K_M and K_cat values shown represent the average of at least 3 independent experiments. Error bars represent the standard deviation.

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with this model, E. coli DinB catalyzes incorporation of dCTP opposite template-2-hydroxyadenine, 2-hydroxyadenine opposite template-dG (or template-dT), and 8-oxo-dG opposite template-dA. If left unrepaired, these insertions will result in TA→GC, GC→TA or AT→CG substitutions, respectively, following the subsequent round of DNA replication [18,51]. Nucleotide sequence analysis indicated that although the majority of H₂O₂-induced mutations corresponded to TA→GC substitutions, this model could also explain the basis for the majority of the mutations within poly-dA sequences that mimic an insertion/deletion loop [52]. It is possible that P. aeruginosa MutS contributes to ROS-induced mutagenesis by influencing the fidelity with which oxidized lesions are repaired. For example, MutS may play an error-prone role in repairing mismatches catalyzed by DinB, particularly mismatches resulting from bypass of oxidized lesions, and/or incorporation of oxidized precursors (such as 2-hydroxy-6-azaadenine and 8-oxo-dG), due to an inability to discriminate between the lesion and the undamaged base, resulting in mutations. Alternatively, MutS might recruit DinB to sites of ROS-induced lesions, contributing to their error-prone bypass. Biochemical approaches are required to test these models.

Epistatic Roles for MutS and DinB

A significant fraction of P. aeruginosa strains isolated from CF airways exhibit a spontaneous hypermutable phenotype due to mutations within mutS [24,25,28,42]. As a result of their elevated spontaneous mutation frequencies, these strains are proposed to acquire adaptive mutations more frequently than those that are MMR-proficient [42,54,53]. Our results indicate that mutS-deficient P. aeruginosa strains are hypersensitive to H₂O₂ (Fig. 2). In addition, we demonstrate that these strains are impaired for H₂O₂-induced mutagenesis (Fig. 3). These results suggest that mutS-deficient strains are ill equipped for colonizing and persisting within the highly oxidizing environment of the early CF airways. Although this view is consistent with the finding that mutS strains are less efficient than wild-type P. aeruginosa at establishing airway infections in mice [28,43], it is nevertheless inconsistent with the
Persisting in the DNA, viability of We further hypothesize that as a result of oxidized lesions would also serve to impair MMR, leading to a mutator phenotype. MutS function would hamper repair of ROS-induced lesions, and MutS, due to finite levels of the proteins involved. Saturation of saturate accurate DNA repair functions, such as those catalyzed by aeroVgna

Airway inflammation contribute to excessive DNA damage in that the remarkably high levels of ROS generated by chronic CF aeroVgna colonizing the CF airways. In this model, we propose damage support an alternative model for pathoadaptation of niches, or under specific conditions, such as end-stage infection. Regardless, these findings in conjunction with our results demonstrating a crucial when airways undergo rapid decline in function. Regardless, these results suggest that mutS-deficient strains may not necessarily have an adaptive advantage over mutS strains in all airway niches. Alternatively, it is possible that hypermutability contributes to adaptation of P. aeruginosa within certain airway niches, or under specific conditions, such as end-stage infection when airways undergo rapid decline in function. Regardless, these findings in conjunction with our results demonstrating a crucial role for MutS and DinB in coping with ROS-induced DNA damage support an alternative model for pathoadaptation of P. aeruginosa colonizing the CF airways. In this model, we propose that the remarkably high levels of ROS generated by chronic CF airway inflammation contribute to excessive DNA damage in P. aeruginosa. We hypothesize that this high level of damage acts to saturate accurate DNA repair functions, such as those catalyzed by MutS, due to finite levels of the proteins involved. Saturation of MutS function would hamper repair of ROS-induced lesions, and would also serve to impair MMR, leading to a mutator phenotype. We further hypothesize that as a result of oxidized lesions persisting in the DNA, viability of P. aeruginosa becomes dependent upon TLS, catalyzed in large part by DinB (see Fig. 3 & Table 3). As TLS is often error-prone, we expect this to contribute further to the mutator phenotype. One advantage of this model over chronic hypermutability is the fact that the mutator phenotype is reversible. As a result, P. aeruginosa can ‘shed’ its mutator phenotype once it has acquired one or more adaptive mutations that serve to protect it from ROS. This ability would minimize the likelihood that members of the P. aeruginosa population would accumulate deleterious mutations. At the same time, our postulated saturation-induced mutator phenotype might contribute to mutations in genes encoding proteins that act in DNA repair, such as mutS, resulting in hypermutable P. aeruginosa strains. Although our model postulates that the induced mutator phenotype contributes to adaptation, it is possible that chronic hypermutability per se does not. In this case, hypermutators may persist by hitchhiking along with truly adaptive mutations, as discussed previously [56]. In conclusion, irrespective of whether an increased mutation frequency is strictly required for P. aeruginosa pathoadaptation in CF, the ability of this pathogen to display a reversible mutator phenotype likely contributes to airway colonization, persistence, and pathoadaptation, particularly under conditions of ROS-induced stress.

ROS-sensitivity of the mutS and/or dinB strains is reminiscent of phenotypes described for P. aeruginosa strains impaired for function of the ‘GO’ component of base excision repair, due to mutations within mutM, mutI, or mutT, which act independently to limit GC→TA transversions resulting from 8-oxo-dG, as well as other oxidized DNA lesions [57,58]. Although P. aeruginosa strains deficient for ‘GO’ function display a level of H2O2 sensitivity that is similar to that observed for the mutS strain, they exhibit significantly increased levels of H2O2-induced mutagenesis, consistent with ‘GO’ function acting to limit ROS-induced mutations [58]. Of relevance to our model discussed above, E. coli MutS is suggested to contribute to MutY function in vivo [37]. Thus, under our model, saturation of MutS impacts not only on error-prone tolerance of ROS-induced lesions and MMR, but may impact on ‘GO’-catalyzed repair of ROS-induced lesions, as well, perhaps leading to an even greater spectrum of mutations that may contribute to pathoadaptation of P. aeruginosa. Collectively, these findings illustrate the complexity of the phenotypes that may be observed with hypermutable P. aeruginosa.
strains, and further highlight the importance of carefully analyzing these strains under a variety of CF-relevant conditions as part of a comprehensive effort aimed at defining the contribution of mutagenesis to *P. aeruginosa* virulence and pathoadaptation.

In summary, findings discussed in this report reveal previously unrecognized roles for MutS and DinB, and additionally uncover a functional relationship between these proteins in modulating the mutation frequency of *P. aeruginosa* following exposure to ROS. Roles for MutS and DinB in ROS-induced mutagenesis are particularly fascinating given the significance of *P. aeruginosa* to CF airway disease. Continued genetic and biochemical characterization of MutS, DinB, as well as additional factors involved in *P. aeruginosa* MMR, TLS, and DNA repair will expand our understanding of these important metabolic pathways, and should provide crucial insights into their contribution(s) to pathoadaptation of this important human pathogen.

### Materials and Methods

#### Commercial reagents and recombinant proteins

Unlabeled ultrapure dNTPs were obtained from GE Healthcare. [γ-32P]-ATP was purchased from M.P. BioMedicals (Irvine, CA). Chemicals were obtained from Sigma-Aldrich. Oligonucleotides used for PCR or nucleotide sequence analysis were synthesized by TriLink Biotechnologies (San Diego, CA). All other oligonucleotides utilized for enzymatic assays, including the template strand oligonucleotide, and subsequent annealing stoichiometric quantities of the primer strand oligonucleotide, and subsequent purification of the duplex template by non-denaturing gel electrophoresis as described [59]. The C-terminally hexahistidine-tagged form of the *P. aeruginosa* DinB protein [20], and the exonuclease-deficient (D219A) bacteriophage T4 DNA polymerase (T4 exonuclease-deficient (D219A) bacteriophage T4 DNA polymerase) was synthesized by TriLink Biotechnologies (San Diego, CA).

#### Bacteriological techniques

*P. aeruginosa* strains used in this study are derived from PAO1, and the salient features of each are detailed in Table 4. Strains were routinely grown in Luria-Bertani (LB; 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) medium [62], unless stated otherwise. When necessary, the following antibiotics were used at the indicated concentrations: rifampicin (Rif), 100 μg/ml; gentamicin (Gent), 100 μg/ml; carbenicillin (Carb), 250 μg/ml; and tetracycline (Tet), 60 μg/ml. *P. aeruginosa* strains MPAO1 (prototroph; identical to PAO1) and MPA2417 (mutS::ISphoA/hah) were obtained from the University of Washington Genome Center [63]. MPA2417 contains an ISphoA/hah transposon inserted after nucleotide 361 (amino acid 121) of the mutS coding sequence (the mutS ORF consists of 2,568 bases, encoding 856 amino acids). The mutS::ISphoA/hah allele in MPA2417 was verified by diagnostic PCR using a protocol provided by Dr. Michael Jacobs of the University of Washington Genome Center. Briefly, primers homologous to either the 5’-PAmutSF; 5’-GAG TGT GGA ACG GAC GAC-3’ or the 3’-end (PAmutSR; 5’-GAG TGT GGA TCC TCA GAG CCG CAT CTT CC-3’) of the mutS gene were paired with a primer homologous to the transposon (hah minus 138; 5’-CGG GTG CAG TAA TAT CGC CCT C-3’) of the mutS gene were paired with a primer homologous to the transposon (hah minus 138; 5’-CGG GTG CAG TAA TAT CGC CCT C-3’). No product was detected using the PAmutSR-hah minus 138 pair, while the expected ~2,300 bp product was observed using the PAmutSR-hah minus 138 pair, verifying both the presence and the correct orientation of the transposon insertion in mutS. Finally, primers PAmutSF and PAmutSR were used to verify the absence of an intact copy of mutS.

*P. aeruginosa* strain UBPA100 (mutS::ISphoA/hah ΔdinB::aacC1) was constructed using the protocol described by Schweizer and colleagues [64]. Briefly, plasmid pLS100 was electroporated into strain MPA32417 (relevant genotype: mutS::ISphoA/hah). Colonies were screened for GentR and sucrose resistance as described previously [65,66]. The presence of the ΔdinB::aacC1 allele was confirmed by PCR analysis using primers PAgent59F (5’-GAG ATG CGC GAC GAC CAC-3’) and PAgent51R (5’-CTG CAG GTC GAG GAG G-3’), which flank the aacC1 cassette located within the dinB gene to amplify an ~2,000-bp fragment consisting of the Gent cassette and flanking dinB sequence. In addition, primers

### Table 4. *P. aeruginosa* strains and plasmid DNAs used in this study.

| Strain | Relevant genotype | Source or construction |
|--------|------------------|------------------------|
| MPAO1  | Prototroph       | [63]                   |
| WFPAA34| ΔdinB::aacC1 (GentR) | [20]                 |
| MPA32417| mutS::ISphoA/hah | [63]               |
| UBPA100| mutS::ISphoA/hah ΔdinB::aacC1 (GentR) | This work |

### Table 4. *P. aeruginosa* strains and plasmid DNAs used in this study.

| Plasmid | Relevant characteristics | Source or construction |
|---------|--------------------------|------------------------|
| pEX18Ap | ApR (CarbR); suicide replacement vector containing *S. subtilis* aacC gene | [65]                |
| pLS100  | ApR (CarbR), GentR; pEX18Ap bearing *P. aeruginosa* ΔdinB::aacC1 (GentR) allele | This work |
| pUCP20T | ApR (CarbR); shuttle vector that replicates in both *P. aeruginosa* and *E. coli* | [69]               |
| pAR101  | ApR (CarbR); pUCP20T bearing *P. aeruginosa* dinB | [20]               |
| pmutS   | ApR (CarbR); pUCP20T bearing *P. aeruginosa* mutS | This work |

*See Materials and Methods for details regarding construction of *P. aeruginosa* strains and plasmids DNAs.

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Determination of spontaneous mutation frequency

*P. aeruginosa* cultures were inoculated from single colonies and grown for 16 hrs in 5 ml of LB containing the appropriate antibiotics. Appropriate dilutions of each culture were plated onto LB plates to determine the number of viable cells, and LB plates were incubated overnight at 37°C. Survival of each strain was calculated relative to a mock-inoculated strain. Corrected ratios were multiplied by the concentration of primer/template used in each assay to determine total product yield. Data obtained for single turnover DNA polymerization assays were fit to equation 1:

\[
y = A(1 - e^{-kt}) + C,
\]

where A is the burst amplitude, k is the observed rate constant \(k_{obs}\) in initial product formation, t is time, and C is a defined constant [59]. Data for the dependency of \(k_{obs}\) as a function of dNTP concentration were fit to the Michaelis-Menten equation (equation 2) to provide values corresponding to \(k_{cat}\) and \(K_M\):

\[
k_{obs} = \left(\frac{k_{cat}}{[dNTP]}\right) / \left(K_M + [dNTP]\right)
\]

where \(k_{obs}\) is the observed rate constant of the reaction, \(k_{cat}\) is the maximal polymerization rate constant, \(K_M\) is the Michaelis-Menten constant for dNTP, and [dNTP] is the concentration of nucleotide substrate [60].

Nucleotide sequence analysis of the *P. aeruginosa* rpoB allele from Rif\(^{B}\) clones

Rif\(^{B}\) *P. aeruginosa* colonies were selected for each strain examined as described above. Eighteen-to-twenty independent colonies for each strain (for spontaneous as well as H\(_2\)O\(_2\)-induced) were grown overnight in LB broth supplemented with Rif. A 250-base pair fragment of the *P. aeruginosa* rpoB gene corresponding to amino acid residues 499-582 was PCR amplified from 2 μl of each culture using primer A1 (5'-GCC TCC TCT GGG CCA TGA GAG CGG GCC AAG ACC GAC AGG AGG-3') and primer B1 (5'-GCG TTT CCA GGG CGC TCA GGG TCC CCA GGG AGT TGA TCA GAC C-3') as described previously [58]. PCR products were purified using the Qiagen Mini-spin kit (Qiagen) as per the manufacturer’s recommendations. The nucleotide sequence of each purified PCR product was determined by the Biopolymers Facility at Roswell Park Cancer Center (Buffalo, NY) using primer A1.

Determination of ultraviolet light (UV), mitomycin C (MMC), and 4-nitroquinoline 1-oxide (4-NQO) -induced mutation frequency

UV-, MMC-, and 4-NQO–induced mutation frequencies were measured as described previously [20]. Values reported represent average of triplicates and were expressed as the percentage of spontaneous mutation frequency.
the frequency of RifR observed following exposure to UV (25 J/m²), MMC (1 mg/ml), or 4-NQO (320 μM) after subtracting the spontaneous RifR frequency for the same strain.

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Author Contributions

Conceived and designed the experiments: MDS LHS AJB DJH. Performed the experiments: LHS BDG JOC SS AJB. Analyzed the data: MDS LHS AJB DJH DJW. Contributed reagents/materials/analysis tools: DJW DJH AJB MDS. Wrote the paper: MDS.

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