Nucleotide excision repair is universally mutagenic and transcription-associated

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Summary

Nucleotide excision repair (NER) is a highly conserved mechanism that removes lesions from DNA. This process has been studied for decades, however, almost all of the work on NER was performed in the presence of exogenous DNA damage. Under these conditions, NER is anti-mutagenic in bacteria. Here, we describe our findings on the role of NER in mutagenesis under endogenous conditions. Counter to dogma, we find that NER is actually pro-mutagenic. Our data suggest a hand-off mechanism between two different types of DNA polymerases that explains the mutagenic nature of NER. Additionally, NER is thought to occur in two different ways; 1) in a transcription-coupled manner where it plays a role in removing lesions that block RNA polymerase, and 2) in a process known as global genome NER, which is independent of transcription. Counter to the classical view, our genetic analyses of the relationship between NER and the RNA polymerase interacting DNA translocase, and evolvability factor, Mfd, indicate that most likely all NER is associated with transcription. Lastly, we show that NER is pro-mutagenic because of endogenous oxidative damage. Altogether, our data strongly suggest that oxidative damage induces a mutagenic NER mechanism, which then accelerates evolution across divergent bacterial species.

Main text

Mutations drive evolution. They provide the necessary genetic diversity that natural selection can then use to help organisms adapt to new environments. Even though mutations are mostly deleterious, and that lower mutation rates are generally accepted to be beneficial, all organisms have a baseline mutation rate that allows them to evolve[1]. However, which endogenous mechanisms most commonly lead to mutations and drive evolution remain poorly understood.

We recently determined that the bacterial RNA polymerase interacting protein Mfd, which is involved in a sub-pathway of the DNA repair pathway nucleotide excision repair (NER), promotes mutations and leads to rapid antibiotic resistance development across highly divergent bacteria[2]. This function depended on Mfd's interaction with the NER protein UvrA. These findings suggested that NER in general might be a mechanism that drives spontaneous mutagenesis in bacteria, potentially even in the absence of transcription. However, this was a hypothesis that would go against dogma. The dogmatic view of NER and its role in reducing mutagenesis was based on a series of studies that utilized artificial DNA damaging conditions. Most *in vivo* studies on NER to date have been performed in the presence of exogenous DNA damage such as UV light. These studies led to the conclusion that NER is an anti-mutagenic DNA repair pathwayfa[3,4]. However, whether this is true in an endogenous context, in unperturbed cells, in the absence of artificial DNA damaging conditions, has not been investigated.

Bacterial NER consists of four steps: 1) Damage recognition, 2) Strand separation, 3) Damaged strand cleavage, 4) Damaged oligonucleotide eviction and 5) Fill in synthesis and ligation[5]. Traditionally, bacterial NER has been thought to consist of two sub pathways that differ only on the recognition step: in transcription-coupled NER (TCR), a stalled RNA polymerase detects the DNA lesion and involves Mfd-dependent recruitment of UvrA; in global genome (GG) NER, lesions are detected by the UvrA dimer and is Mfd independent[5,6]. Downstream of damage recognition, UvrB separates the strands and guides the UvrC nuclease to cleave the damaged strand. The damage containing oligo is removed by the UvrD helicase and the pathway is fished by gap filling DNA synthesis[5]. A recent alternative model for damage recognition during TC-NER suggests that UvrD instead of Mfd recruits the NER factors and proposes that, in bacteria, all NER is transcription coupled, although these conclusions remain somewhat controversial[7–10].

Here we show that the core NER factors UvrABC promote spontaneous mutagenesis, across highly divergent bacterial species. We also show that a replicative polymerase, and two Y-family polymerases are responsible for the observed NER-dependent increase in mutagenesis. Critically, our genetic analyses show that all three polymerases function in the same pathway as the NER proteins and Mfd. These findings strongly suggest that bacterial NER is a pro-mutagenic repair pathway that is universally coupled to transcription, and that this is due to the usage of error prone polymerases. Last, we present data that the underlying cause of this process is endogenous oxidative stress and that in general, oxidative damage drives NER mutagenesis and rapid evolution in bacteria.

NER promotes mutagenesis in bacteria

We and others have previously shown that, in the absence of exogenous DNA damage, the bacterial transcription-coupled repair (TCR) protein Mfd promotes mutagenesis across many different bacterial species[2,11,12]. In addition, we previously showed that this pro-mutagenic effect depends on the interaction of Mfd with the RNA polymerase (RNAP) and the NER protein UvrA[2]. This brings up the possibility that NER, which is thought to promote genome stability and reduce mutagenesis[3,4], is pro-mutagenic in the absence of exogenous DNA damage, in the endogenous context.

We first tested this hypothesis by measuring mutation rates using the Luria-Delbruck fluctuation assays[13] in wild-type *Bacillus subtilis* strains and isogenic strains that lack each of the NER proteins: UvrA, UvrB and UvrC. We performed these experiments in the absence of exogenous DNA damage. Strikingly, we observed a 50-75% decrease in the mutation rates in NER deficient strains (Fig. 1A).

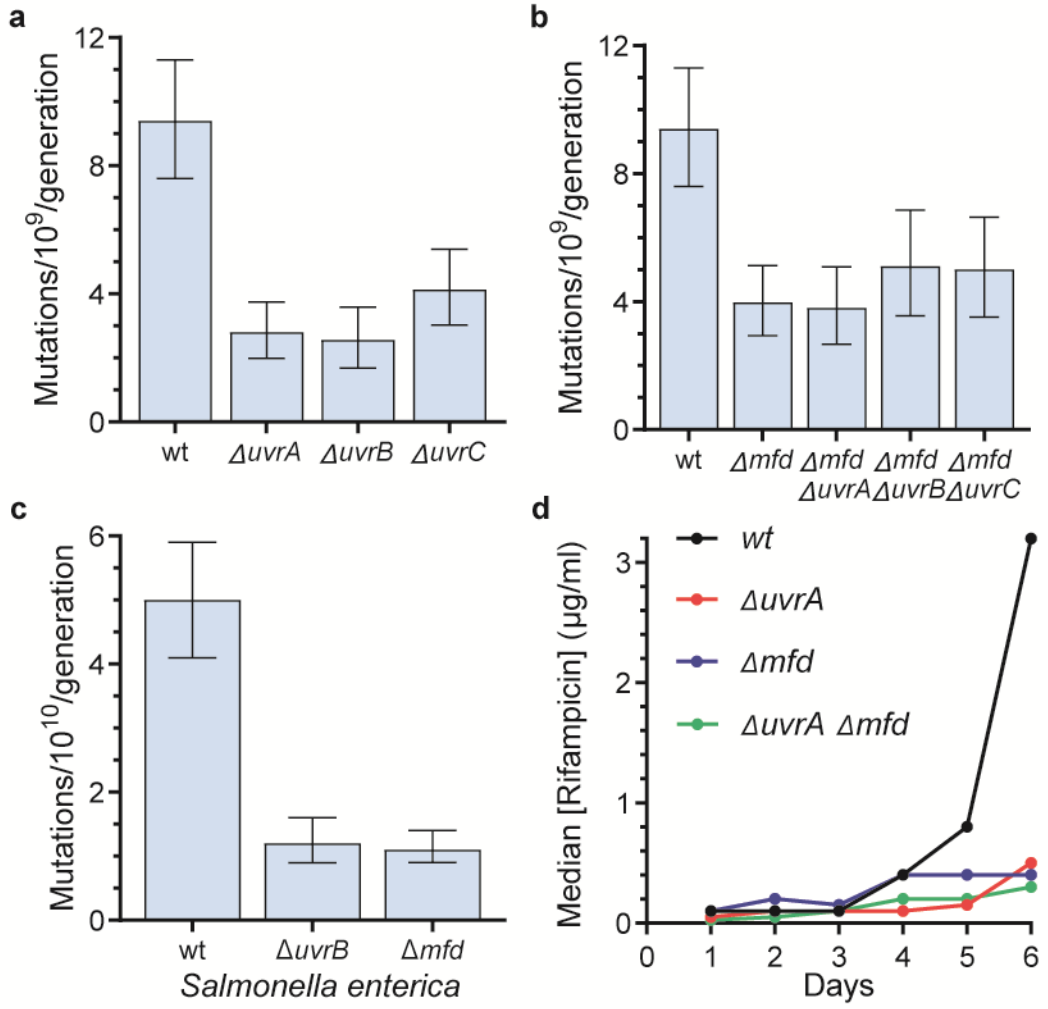

Figure 1: a, b) Mutation rates of *Bacillus subtilis* strains measured using rifampicin. n=54 (wt), 48 (Δ*uvrA*), 37 (Δ*uvrB*), 48 (Δ*uvrC*), 59 (Δ*mfd*), 40 (Δ*mfd* Δ*uvrA*), 40 (Δ*mfd* Δ*uvrB*), 50 (Δ*mfd* Δ*uvrC*) biological replicates. **c)** Mutation rates of *Salmonella enterica* serovar Typhimurium strains measured using rifampicin. n=54 (wt), 40 (Δ*uvrB*), 48 (Δ*mfd*). **d)** Median rifampicin concentration that allows for

growth in the indicated strains at the indicated timepoints. n=23 (wt), 24 (Δ*uvrA*), 12 (Δ*mfd*), 12 (Δ*uvrA* Δ*mfd*). Error bars are 95% confidence intervals.

To determine whether the pro-mutagenic effects of NER were due to TCR and/or GG-NER, we built double mutants that lacked Mfd and the Uvr proteins. If NER is mutagenic due to only TCR, then we expected that the double mutants lacking Mfd and NER proteins would have an epistatic relationship, and that the mutation rates of the double mutants would be similar to the single mutants. On the other hand, if NER-mediated mutagenesis is through both GG-NER and TCR, the combination of mutants lacking both Mfd and NER proteins would further reduce mutation rates. To discern between these possibilities, we measured and compared the mutation rates of the single and double mutants side-by-side. We found that the mutation rates of strains lacking both Mfd and all three canonical NER factors have the same mutation rates as each single mutant alone (Fig. 1B). This strongly suggests that all (mutagenic) NER is coupled to transcription.

To determine whether the mutagenic nature of NER is conserved across bacterial species, we next determined the mutation rates of similar strains in *Salmonella enterica* serovar Typhimurium, a Gram-negative bacterium which is highly divergent from *B. subtilis.* When we compared the mutation rates of wild-type strains to those that either lack a Uvr protein or Mfd, we found results that were consistent with what we observed in *B. subtilis*. In the absence of UvrB, there was a similar decrease in mutation rates compared to strains lacking Mfd. These results indicate that, the mutagenicity of NER is conserved amongst bacteria (Fig. 1C).

We have shown in the past that, over time, relatively small (in this case a 50-75% decrease) differences in mutation rates can translate into large differences in the rate of evolution[2]. We therefore used a laboratory evolution assay we previously established to determine whether the mutagenic nature of NER accelerates evolution. For this, we measured adaptation to the transcription inhibitor rifampicin in wild-type strains to those lacking UvrA or Mfd, or both, in *B. subtilis*[2]. We have previously shown that the increase in the minimal inhibitory concentration (MIC) observed over time correlates with the appearance of mutations in known resistance genes[2]. After six days (approximately 40 generations), the median concentration of rifampicin wild-type cells were able to survive was 3.2 µg/ml, while this was markedly lower for *uvrA* null (0.4 µg/ml), *mfd* nulls (0.5 ug/ml), and *uvrA mfd* double mutants (0.3 µg/ml) (Fig. 1D). This indicates that the differences observed in the mutation rates in NER deficient cells are compounded and ultimately have large effects on adaptive evolution. In addition, these results support the observation that all mutagenic NER is in the same pathway as Mfd, and again, most likely universally transcription dependent.

A replicative and two Y-family polymerases function in the same pathway as NER

NER is generally thought to be an error free pathway. However, our observations clearly suggest that this is not the case in the endogenous context. Our data suggest that the gap filling step of NER is completed by an error-prone mechanism. During NER, gap-filling synthesis is the last step of the pathway and based on *in vitro* experiments that examined DNA polymerase I (PolA in

B. subtilis), the dogmatic view became that this is the polymerase that functions during this step[5]. Thus, we measured mutation rates in cells lacking PolA. Although *in vitro* work had led to the conclusion that PolA is a high-fidelity polymerase[14], we found that this is not the case *in vivo*. We observed that PolA is mutagenic, as cells lacking PolA showed a decrease in mutation rates that were very similar to that seen in NER deficient strains.

To determine whether NER is mutagenic due to PolA activity, we measured the mutation rates of *uvrA polA* and *mfd polA* double knockouts. When we compared the mutation rates of strains lacking either PolA, Mfd, or UvrA alone to the double mutants that lacked Mfd and PolA as well as UvrA and PolA, we did not see an additional decrease in mutation rates, indicating that Mfd-associated, mutagenic NER is in the same pathway as PolA (Fig. 2A). In addition, we used a biochemical assay where we purified *B. subtilis* PolA and used an *in vitro* primer-extension assay on a ssDNA gap template similar to the one that would be generated during NER to re-examine whether PolA can fill in this gap. We indeed observed that PolA is able to efficiently fill in this gap (Extended Data Fig 1A, B).

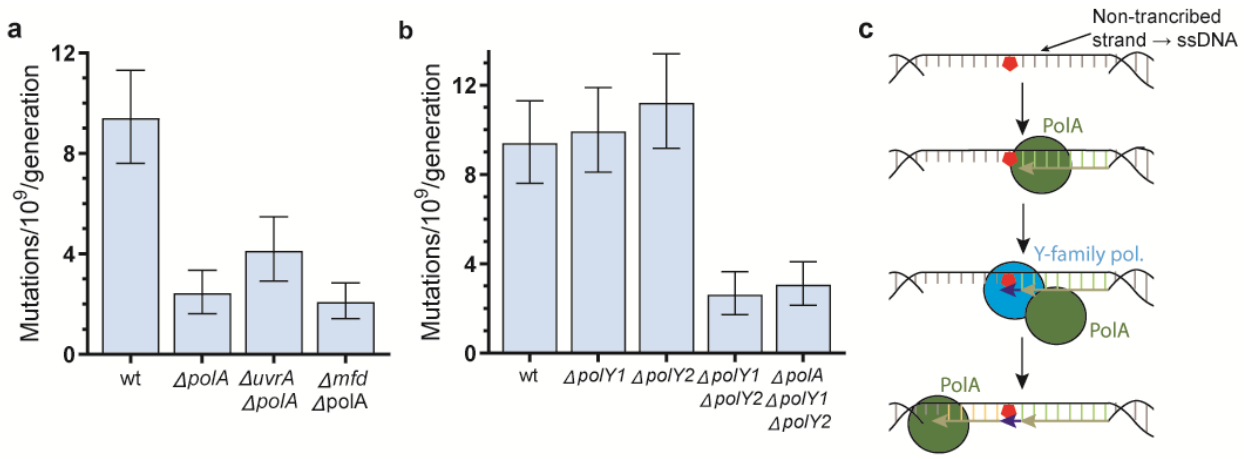

Figure 2: a, b) Mutation rates of *B. subtilis* strains. n=54 (wt), 40 (Δ*polA*), 36 (Δ*uvrA* Δ*polA*), 44 (Δ*mfd* Δ*polA*), 57 (Δ*polY1*), 56 (Δ*polY2*), 35 (Δ*polY1* Δ*polY2*), 43 (Δ*polA* Δ*polY1* Δ*polY2*). **c)** Model for the molecular mechanism of NER-dependent mutagenesis. Due to DNA being single stranded in the transcription bubble and/or during NER, the non-transcribed strand is prone to damage that stalls PolA and leads to the recruitment of Y-family polymerases, further increasing the possibility of acquiring a mutation. Error bars are 95% confidence intervals.

However, *in vitro* studies with the *E. coli* ortholog of PolA (PolI) have determined that it is a high-fidelity polymerase, making it unlikely that by itself, it would introduce an error in such a small gap as the one generated during NER[14]. Given that previous work has suggested that *B. subtilis* PolA interacts with two error-prone, Y-family polymerases, PolY1 and PolY2 (orthologs of the *E. coli* DinB and UmuC and the mammalian Pol kappa and Pol eta)[15], we reasoned that these Y-family polymerases could also be involved in the pro-mutagenic nature of NER. To test our model, we generated strains that lacked either PolY1, PolY2, or both polymerases. When we determined the mutation rates of strains that either lacked PolY1 or PolY2, we didn't observe a

decrease in mutation rates in either single mutant. Interestingly we did observe a decrease in mutation rates in strains lacking both PolY1 and PolY2, suggesting a redundant, pro-mutagenic role for these polymerases (Fig 2B). If our hypothesis that these polymerases cooperate with PolA during the NER gap filling step is correct, then in strains that lack all three polymerases, we should not observe any additional decrease in mutation rates. Indeed, we observed that there was no additional decrease in mutation rates when cells lacked all three polymerases compared to cells either lacking only PolA, the Uvr proteins, or both PolY1 PolY2 (Fig 2B and Extended Data Fig 1B). Therefore, we conclude that these polymerases are in the same pathway and cooperate to complete the last step during NER.

The observed requirement for both an A-family replicative polymerase and a Y-family polymerase led us to the hypothesis that PolA performs DNA synthesis during NER, but that it often stalls at a DNA lesion that is present on the opposite strand to the original lesion that was excised. This stalled PolA would then recruit a Y-family polymerase to overcome this lesion, further increasing the chances of generating a mutation. More specifically, this DNA lesion would be on the NER template strand, which is the non-transcribed strand. Therefore, it is possible that the origin of this secondary damage stems from the non-transcribed strand being single stranded during transcription and/or NER, which would make it more susceptible to DNA lesions (Fig 2C)[16,17].

To test this model, we again turned to our biochemical assay where we used purified PolA and measured DNA synthesis on a template where we introduced an abasic site on the opposite strand to that which is removed by the NER protein UvrD. We observed that an abasic site, one the most common lesions observed in DNA[18], is a strong block to synthesis by PolA[9] (Extended data Fig 1C), supporting the model that PolA alone cannot fill in a gap generated during NER that has damage on the non-transcribed strand. To complete gap-filling at this stage, Y-family polymerases are most likely required, consistent with the results described above.

Oxidative stress drives NER-dependent evolution

In this study, all mutation rates and evolution assays were performed in the absence of exogenous DNA damage. Thus, we wondered what the endogenous source of DNA damage behind NER mutagenesis is that damages the non-transcribed strand. Because oxidative damage is considered the most common endogenous form of DNA damage[19], and it damages ssDNA much more efficiently than dsDNA[16,17], we tested if oxidative DNA damage is responsible for NER being pro-mutagenic and a strong driver of evolution.

To test our model, we performed evolution assays where we decreased the levels of oxidative stress that the cells experience using two different methods. First, we performed evolution assays in the presence of the antioxidant thiourea. This is an antioxidant molecule that has been used in the past to reduce oxidative stress in bacteria[20–22]. We used concentrations that do not affect the growth rate of our strains (Extended Data Fig 2A). We observed a marked decrease in the rate of evolution of wild-type cells to various antibiotics when thiourea was present in the media (Fig. 3A, Extended Data Figure 2B). To confirm that the effect we observed was not simply due to some artifact generated by thiourea, we also made a *B. subtilis* strain that overexpresses *katA*, the

gene that codes for the vegetative catalase, which breaks down hydrogen peroxide[23]. Similar to the results we obtained with thiourea, we observed a decrease in the rate of evolution to the antibiotic rifampicin (Fig. 3A). These results together strongly suggest that oxidative DNA damage is indeed driving evolution.

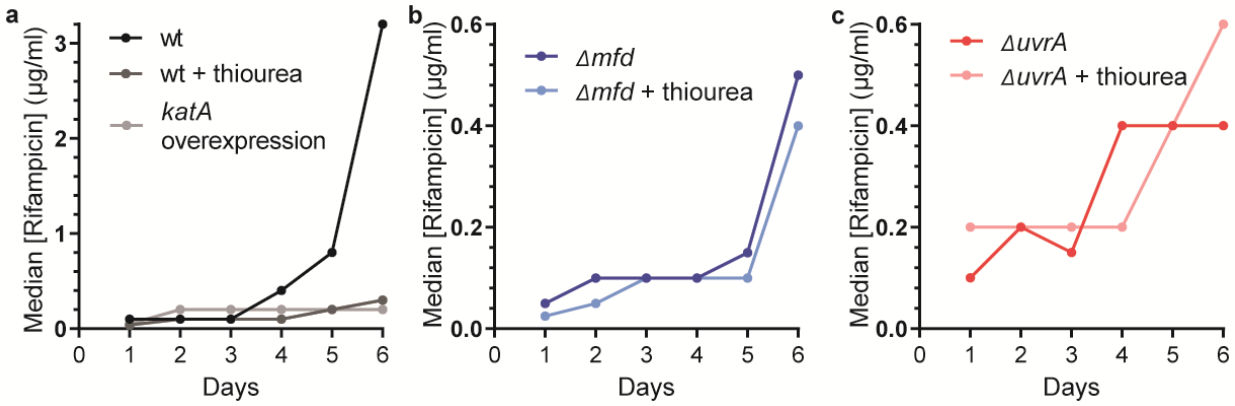

Figure 3: a, b, c) Median rifampicin concentration that allows for growth in the indicated strains at each sampled timepoint. 50 mM thiourea was included in the media where indicated. 1mM IPTG was added for *katA* overexpression. n=23 (wt - thiourea), 12 (wt + thiourea), 12 (Δ*mfd* - thiourea), 12 (Δ*mfd* + thiourea), 24 (Δ*uvrA* - thiourea), 12 (Δ*uvrA* + thiourea) biological replicates.

For both thiourea and *katA* overexpression, the decrease in the rate of evolution was similar to those observed in strains lacking NER proteins. We therefore tested whether oxidative damage is responsible for the mutagenic nature of NER. We performed evolution assays in strains deficient in NER genes (*uvrA* nulls and *mfd* nulls). Although these strains have a serious deficiency in evolving resistance to antibiotics, towards the end of the evolution assays, a slight increase in their MIC can be observed (Fig. 3B, C). We took advantage of this and analyzed the rate at which evolution starts to take off at the last time points when oxidative stress is reduced. Consistent with our model, we observed that, in strains lacking Mfd, or the NER proteins, or both, thiourea didn't have any effect on the rate of evolution. This strongly suggests that NER mutagenesis and subsequent adaptive evolution depends on endogenous oxidative stress.

Last, to determine whether this was a conserved mechanism, we performed evolution assays in patient derived strains of *Pseudomonas aeruginosa* (CF127)[24] and multidrug resistant *Staphylococcus aureus* (MRSA). Again, we performed evolution assays, with and without thiourea, at a concentration that again doesn't affect the growth rate of these bacterial species (Extended Data Fig 2C, D). Consistent with our observations in *B. subtilis*, we found that in these other species, there was a significant decrease in the rate of evolution when thiourea was included in the media (Fig 4A, B). This effect is particularly striking in the case of the MRSA strain, for which the concentration of antibiotic in which it could survive increased ≈250,000X (Fig 4B) in the absence of thiourea. This increase was 32X for *B. subtilis* (Fig 3A) and ≈250X for *P. aeruginosa* (Fig 4A).

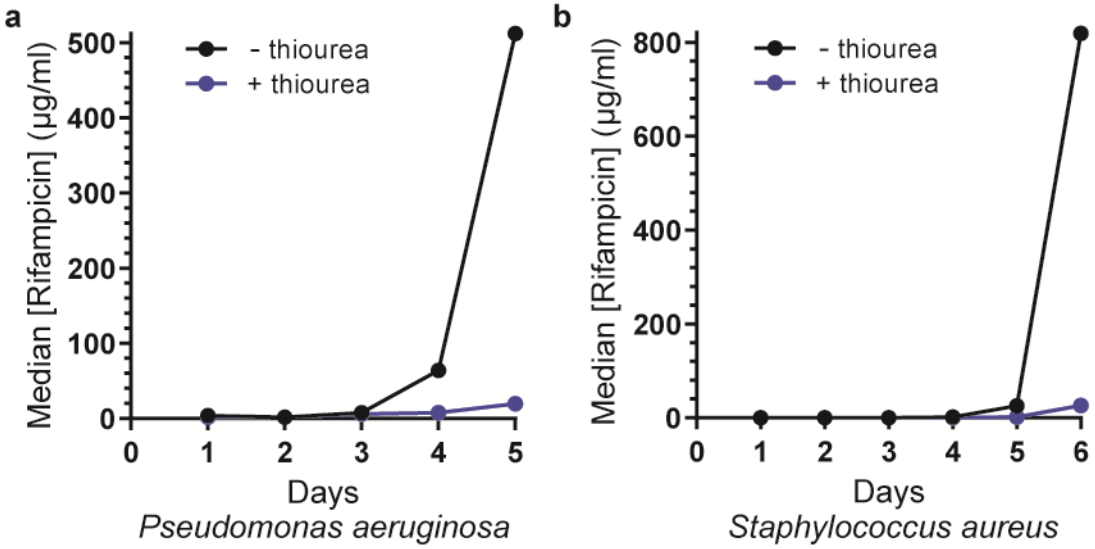

Figure 4: a, b) Median rifampicin concentration that allows for growth in *Pseudomonas aeruginosa* (CF127) (a) and *Staphylococcus aureus* (b) at each sampled timepoint. 10 mM (*P. aeruginosa*) or 50 mM (*S. aureus*) thiourea was included in the media where indicated. n=12 biological replicates for all strains.

Discussion

We have shown that nucleotide excision repair (NER), which strongly suppresses mutagenesis in cells exposed to DNA damaging agents[3,4], is actually promoting mutagenesis under endogenous conditions and is generally a pro-mutagenic mechanism. Bacteria lacking any one of the three core components of the NER mechanism, UvrABC, have lower mutation rates than wild-type cells, indicating that NER causes spontaneous mutations (Fig. 1A, C). In addition, our data indicate that all mutagenic NER functions in the same pathway as the transcription-coupled (TC) NER factor Mfd, suggesting that NER is universally transcription dependent, at least under endogenous conditions (Fig. 1B). This is consistent with recent biochemical findings regarding NER and transcription and brings into question whether GG-NER is a mechanism that exists[10].

Interestingly, our data show that it is the cooperative nature of at least two DNA polymerases that causes NER-dependent mutations: the replicative polymerase commonly associated with NER, PolA, and one of two redundant Y-family polymerases, PolY1 and PolY2 (Fig. 2A-C, Extended Data Fig 1C). We propose that DNA damage in the NER template strand (the non-coding strand, Fig 2C) explains this requirement for both DNA polymerases to complete NER, which will naturally lead to an increased likelihood of mutations being introduced into the synthesized DNA gap. This DNA lesion would be independent of the lesion that triggered NER, and we propose that it is caused during transcription and NER, as the non-transcribed strand stays as ssDNA for an extended period of time during both processes and it is well-known that ssDNA is more prone to damage than dsDNA[16,17].

There is a potential alternative model: the NER machinery has been shown to excise non-damaged DNA *in vitro*[25] and that transcription stimulates this process *in vivo*[26]. These gratuitous repair events, even if much less efficient than excision of damaged DNA, are predicted to be a common phenomenon, as the amount of non-damaged DNA outweighs the amount of damaged DNA by several orders of magnitude in cells that are not exposed to exogenous DNA damaging agents[27]. Moreover, Mfd has been found bound to DNA throughout the genome in the absence of exogenous DNA damage[28,29], and it plays a role in transcription that is independent of its role in TC-NER[30]. This constitutive association with DNA and RNAP could lead to excision, fill in synthesis, and therefore increasing the likelihood of mutations being introduced onto undamaged DNA.

Interestingly, a recent pre-print has proposed that DNA lesions that are in close proximity and on different strands lead to mutagenic NER, using a mouse liver cancer model in which cells are exposed to high levels of the DNA damaging agent diethylnitrosamine[31]. Moreover, NER has been found to be pro-mutagenic in stationary phase yeast cells irradiated with UV light[32–35]. Several models have been proposed that could explain this, including damage to both strands of the DNA, which is supported by the requirement of proteins involved in error-prone bypass of DNA damage in NER-dependent mutagenesis[36]. These findings, together with our results, suggest that the mechanism of NER-induced mutagenesis is conserved from bacteria to mammals. Our findings add an additional piece of information: NER is universally coupled to transcription.

Last, we identify endogenous oxidative stress as the main source of NER-dependent mutagenesis and evolution. Oxidative stress is an obligatory consequence of aerobic life, and it results from an imbalance of reactive oxygen species. Reactive oxygen species have been shown to lead to most spontaneous mutagenesis in *E. coli*[37] and are thought to be an important source of endogenous DNA damage[38]. We tested the contribution of oxidative stress to mutagenesis by measuring the evolution of resistance to antibiotics in cells that were grown in the presence or absence of thiourea, an antioxidant that has been shown to reduce oxidative stress in bacteria[20]. We observed that the evolution of antibiotic resistance was much slower when thiourea was present in the media for both *B. subtilis* and patient-derived pathogenic strains of *P. aeruginosa* and MRSA (Fig 3A, Fig 4), indicating that oxidative stress drives evolution. Moreover, we observed a similar effect when we overexpress the vegetative catalase *katA* (Fig. 3A), which breaks down hydrogen peroxide, a molecule that can't itself react with DNA, but that can react with free cellular iron which oxidizes DNA[38]. Interestingly we observed that the little evolution we observe in NER deficient strains is *not* diminished in the presence of thiourea, supporting our model that NER-dependent mutagenesis is mostly due to lesions caused by oxidative stress. This is consistent with the mutagenic footprint of NER observed in mammalian cells[31]. Therefore, in addition to showing that NER is universally mutagenic and transcription-associated in bacteria, our findings likely explain the main source of NER-dependent mutagenesis not only in bacteria, but also in higher eukaryotes.

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Methods

Bacterial culture

Bacillus subtilis, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* were cultured in lysogeny broth (LB), and *Pseudomonas aeruginosa* in LB with 0.1% tween 20 (when liquid media). Bacterial plates were grown overnight at 37 °C unless otherwise indicated with the following antibiotics when appropriate: 500 µg/ml erythromycin and 12.5 mg/ml lincomycin (MLS), 5 µg/ml (*B. subtilis*) or 50 µg/ml (*E. coli* and *S. enterica*) kanamycin, 25 µg/ml chloramphenicol and 100 µg/ml carbenicillin. When grown in liquid media, cultures were started from single colonies and were grown with aeration (260 rpm).

Strain construction

The parental strain for all *B. subtilis* strains used in this study is HM1 (same as AG174, originally named JH642)[39,40]. Gene deletions that are marked with MLS or kanamycin resistance were obtained from[41]. Genotypes for all strains used can be found in Extended Data Table 1. Genomic DNA from these strains was purified with the GeneJET Genomic DNA Purification Kit

(Thermo) following the manufacturer's instructions and transformed into the HM1 background as in previously decribed[42]. When necessary to make strains that carry multiple mutations, these antibiotic resistant cassettes were excised by transforming the strains with a plasmid expressing the Cre recombinase (pDR244, BGSCID: ECE274) purified from RecA+ *Escherichia coli* (NEB) cells with the GeneJET Plasmid Miniprep Kit (Thermo), generating markerless strains[41]. Recombinants containing markerless strains were checked by PCR (Extended Data Table 2).

The *S. enterica* Typhimurium strain is SL1344[43] and was a gift from Mariana Byndloss (Vanderbilt University), the *Pseudomonas aeruginosa* strain is CF127[24] and was a gift from Matt Parsek (University of Washington) and the multidrug-resistant *Staphylococcus aureus* strain is a cystic fibrosis patient derived strain obtained from the Vanderbilt University Medical Center.

For *katA* overexpression, the coding sequence of the *katA* gene was amplified using Q5 polymerase (NEB) (Extended data Table 2) and cloned between the *Hind*III and the *Nhe*I sites in pCAL838[44] to form pHM724. pHM724 DNA obtained from RecA+ *Escherichia coli* cells was transformed into HM1 cells. Cells were plated on MLS containing plates and after overnight incubation at 37 °C, MLS resistant colonies were tested for growth in media lacking threonine. Colonies that lack growth in threonine less media and were MLS resistant were selected as integrants.

For *S. enterica*, knock outs were made by recombineering as previously described[45] using the pSIM27 plasmid, a gift from the Court lab (https://redrecombineering.ncifcrf.gov/strains--plasmids.html). In short, for knocking out *mfd*, the chloramphenicol resistance gene was amplified from the pKD3 plasmid (a gift from the Wanner lab[46]) while adding 40 nucleotides of homology upstream of the start site and downstream of the stop codon using Q5 polymerase (Extended Data Table 2). The PCR amplicon was cleaned and electroporated into competent, wt cells harboring the pSIM27 plasmid. Chloramphenicol resistant colonies were selected and checked by PCR (Extended Data Table 2). For knocking out *uvrB*, the kanamycin resistance gene was amplified from an *E. coli* strain with this gene on its chromosome (Extended Data Table 2).

Determination of the mutation rates by fluctuation assays

Mutations rates were calculated as previously described[2]. A single colony was inoculated into 2 ml of LB and grown for 2 hours (*B. subtilis*) or 2.5 hour (*S. enterica*) to reach exponential growth (0.1 <OD<0.6). This culture was diluted to an OD of 0.0005 and between 3 and 10 parallel cultures with 2 ml of LB were grown for 4.5 hours. Then, 1.5 ml of cells were pelleted and plated on 50 ug/ml rifampicin containing plates. The remaining cells were serially diluted in 1X Spizizen media and plated on antibiotic free media to quantify total viable cells. Colonies were counted after 24 hours at 37 °C (rifampicin plates) or 16 hours at 30 °C (no antibiotic plates). Mutation rates were calculated by using the Fluctuation AnaLysis CalculatOR[47], utilizing the Ma-Sandri-Sarkar maximum likelihood method.

Evolution assays

Evolution assays were performed as previously described[2]. A single colony of the indicated species and genotype was grown until and OD of 1-2 was reached. Culture was then diluted to and OD of 0.01 in culture media and grown in 7 different concentrations of the indicated antibiotic, ranging from no antibiotic to 16X the minimal inhibitory concentration (MIC), as well as thiourea when indicated. Cells were grown for 24 hours at 37 °C with aeration, after which the OD was measured. The culture with the highest antibiotic concentration that showed an OD larger than 0.5X the OD of the culture without antibiotic (or, in the case of P. aeruginosa, an OD>0.3) was diluted 100X to an OD of approximately 0.01 and again grown in 7 different antibiotic concentrations. This process was repeated 6 times for *B. subtilis* and *S. aureus* or 5 times for *P. aeruginosa*.

Growth curves

Growth curves were determined by growing a single colony of the indicated species until and OD of 1-2 was reached. The culture was diluted to an OD of 0.01 in culture media and growth in an Epoch microplate spectrophotometer (BioTek) at 37 °C for 16 hours. OD600 was measured every 10 mins.

PolA purification

The coding sequence of PolA without the start codon was amplified by PCR using Q5 polymerase (NEB) and cloned BamHI-XhoI into pET28a (Thermo) to generate an N-terminal 6X his tagged protein coding sequence. The plasmid was transformed into BL21(DE) cells (NEB), and a single colony was inoculated into 70 ml of LB and grown overnight in LB containing kanamycin. 10 ml of culture were then inoculated in 1 L of LB+kanamycin and grown until an OD600 of 0.6, when 1 mM IPTG was added to the media. Cells were grown for 4 hours and centrifuged for 15 mins at 4000G. Pellets were resuspended in 30 ml of CelLytic B cell lysis reagent (Sigma) with 3 µl of Benzonase (Sigma) and 10 mM imidazole and shaken at RT for 10 mins. Lysate was centrifuged at 20000G at 4 °C and the supernatant was mixed with an equal volume of equilibration buffer (20 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 10 mM imidazole), and run twice through 15 ml of equilibrated HisPur™ Ni-NTA Resin (Thermo) at 4 °C. Resin was washed with 150 ml of wash buffer (20 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 40 mM imidazole) and eluted with 15 ml of elution buffer (20 mM sodium phosphate pH 7.4, 300 mM sodium chloride, 150 mM imidazole). Protein was dialyzed with a 30 ml Slide-A-Lyzer Dialysis Cassette G2 20000 MWCO (Thermo) against 10 mM tris pH 8, 50 mM NaCl, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA for 3 hours at RT. Protein prep was then concentrated with Amicon Ultra-15 Centrifugal Filter Units 3000K (Millipore) to a final concentration of 1.6 mg/ml measured by Bradford assay (Thermo). PolA prep was run on a 10% SDS-PAGE and stained by Imperial protein stain (Thermo) to confirm purity of purified enzyme.

PolA synthesis assay

PolA synthesis was tested on 40 mM Tris pH 8, 10 mM $MgCl_2$, 60 mM KCl, 2.5% glycerol buffer containing 1 mM dNTPs, 1.5 nM of the indicated DNA substrate labeled with Cy5, and 100 nM PolA. 10 ul reactions were incubated at 37 °C for 30 mins and stopped with 10 ul of

95% formamide 10 mM EDTA. DNA was denatured at 85 °C for 15 mins and run in a 12% urea denaturing gel at 150V for 30 mins. Gel was scanned in a ChemiDoc imaging system (BioRad).

The substrates for PolA synthesis experiments were done by annealing three (gap substrate) or two (abasic site substrate) HPLC purified oligos (Sigma) in a thermocycler. The template for the abasic site substrate contained a deoxyuracil in the 9th position. The abasic site was generated by treating the annealed oligo with hSMUG1 (NEB) for 30 mins at 37 °C followed by heat inactivation of the enzyme at 65 °C for 20 mins.

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Author Contributions and Affiliations

JCG ANS and AJHV performed experiments. JCG and ANS analyzed data. JCG created figures. JCG and HM wrote the manuscript. HM directed the project.

Competing interest declaration

The authors declare no conflict of intertest

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Extended data

Extended Data Table 1: Strains used

Strain	Species	Genotype	Reference	Figure
HM1	*B. subtilis*	wt	Brehm 1973	1, 2, 3, S2
HM2521	*B. subtilis*	*mfd::MLS*	Million-Weaver 2015	1,3
HM2633	*B. subtilis*	*uvrA::MLS*	This study	1,3
HM2634	*B. subtilis*	*uvrB::MLS*	This study	1
HM2635	*B. subtilis*	*uvrC::MLS*	This study	1
HM2472	*B. subtilis*	*mfd::markerless uvrA::MLS*	This study	1
HM2473	*B. subtilis*	*mfd::markerless uvrB::MLS*	This study	1
HM2474	*B. subtilis*	*mfd::markerless uvrC::MLS*	This study	1
HM4315	*S. enterica*	wt	Hoiseth and Stocker 1981	1
HM4500	*S. enterica*	*mfd::Cm*	This study	1
HM4510	*S. enterica*	*uvrB::Kan*	This study	1
HM3533	*B. subtilis*	*polA::MLS*	This study	2
HM4449	*B. subtilis*	*uvrA::markerless polA::MLS*	This study	2
HM3550	*B. subtilis*	*mfd::markerless polA::MLS*	This study	2
HM391	*B. subtilis*	*polY1::Cm*	Million-Weaver 2015	2
HM345	*B. subtilis*	*polY2::Cm*	Million-Weaver 2015	2
HM2632	*B. subtilis*	*polY1::MLS polY2::Cm*	This study	2
HM3567	*B. subtilis*	*polY1::markerless polY2::Cm polA::MLS*	This study	2
HM2666	*B. subtilis*	*polY1::markerless polY2::Cm uvrA::MLS*	This study	S1
HM2667	*B. subtilis*	*polY1::markerless polY2::Cm uvrB::MLS*	This study	S1
HM2668	*B. subtilis*	*polY1::markerless polY2::Cm uvrC::MLS*	This study	S1
HM2669	*B. subtilis*	*polY1::markerless polY2::Cm mfd::MLS*	This study	S1
HM4488	*B. subtilis*	*polY1::markerless polY2::Cm*	This study	S1

		mfd::markerless polA::MLS		
HM4482	*B. subtilis*	*polY1::markerless polY2::Cm uvrA::markerless polA::MLS*	This study	S1
HM4502	*B. subtilis*	*thr::Pspank(hy) katA*	This study	3
HM2212	*P. aeruginosa*	*CF127*	Wolfgang 2003	4, S2
HM4318	*S. aureus*	penicillin, oxacillin, erythromycin resistant	This study	4, S2

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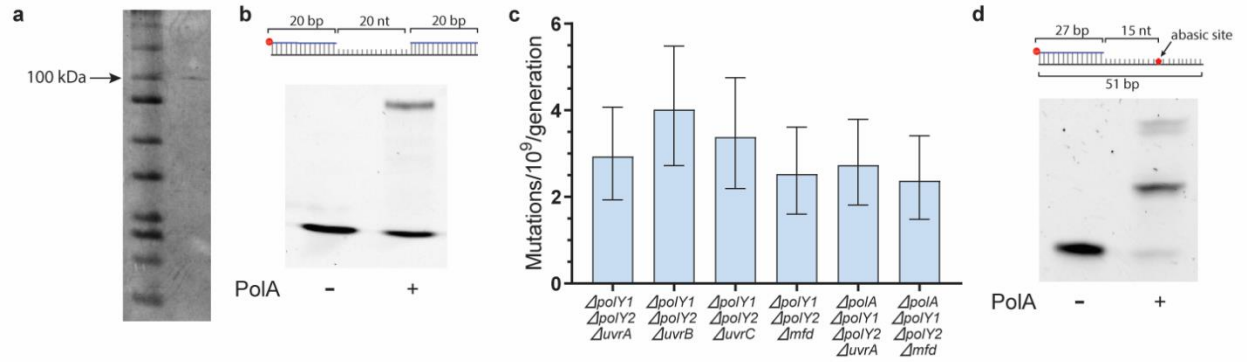
528 Extended Date Table 2: Oligonucleotides used

PCR/substrate	Species	Oligo	Sequence (5'->3')
uvrA::markerless	*B. subtilis*	Fwd	GGAGCTTCGCGATTTACTTTTAG
		Rev	GCTTGCCTGCTAAGCCC
mfd::markerless	*B. subtilis*	Fwd	CGAAATCCGCATTACCACGA
		Rev	TTAGGAATCACGACCCGACC
polY1::markerless	*B. subtilis*	Fwd	TGTTACGGCGCTGTGTATC
		Rev	CGAATTCATGCGGAAGACTTTAC
uvrB recombineerimg	*S. enterica*	Fwd	TACACCCCTGCCCGCTCACTCCTTCAGGT AGCCGCTCATGTATGGACAGCAAGCGAAC CG
		Rev	CCATGGTAACGATGACTCGCTGGCGATCG ACACATTGTCATCAGAAGAACTCGTCAAG AAG
mfd recombineerimg	*S. enterica*	Fwd	GACGCCCGGCCTGACGCTTATGCAATAGC GTTTTCTTCCAGTGTAGGCTGGAGCTGCTT C
		Rev	GTGCGGCGTAAAACAAAAAGAGATACTG ACAACCGTTATGCATATGAATATCCTCCT TAG
uvrB check	*S. enterica*	Fwd	GCAATATTCACCGTCGAGAG
		Rev	CTATTGCACTGAAATTCTCAAAAGC
mfd check	*S. enterica*	Fwd	AGAATTTGTAAAGATTAGGCCGG
		Rev	TGAAGCAGCCTGAAGGG
Gap substrate	*In vitro*	Top left	GCCTAGCTCTGCCATGCATA
		Top right	TACACCTGTCTATCATTAGT
		Bottom	ACTAATGATAGACAGGTGTAGTACGGAA ATCTTCTACGTTTATGCATGGCAGAGCTA GGC
Abasic site substrate	*In vitro*	Top	Cy5-ATTCTGGTGGAAATGGCGCGCTGCTAT

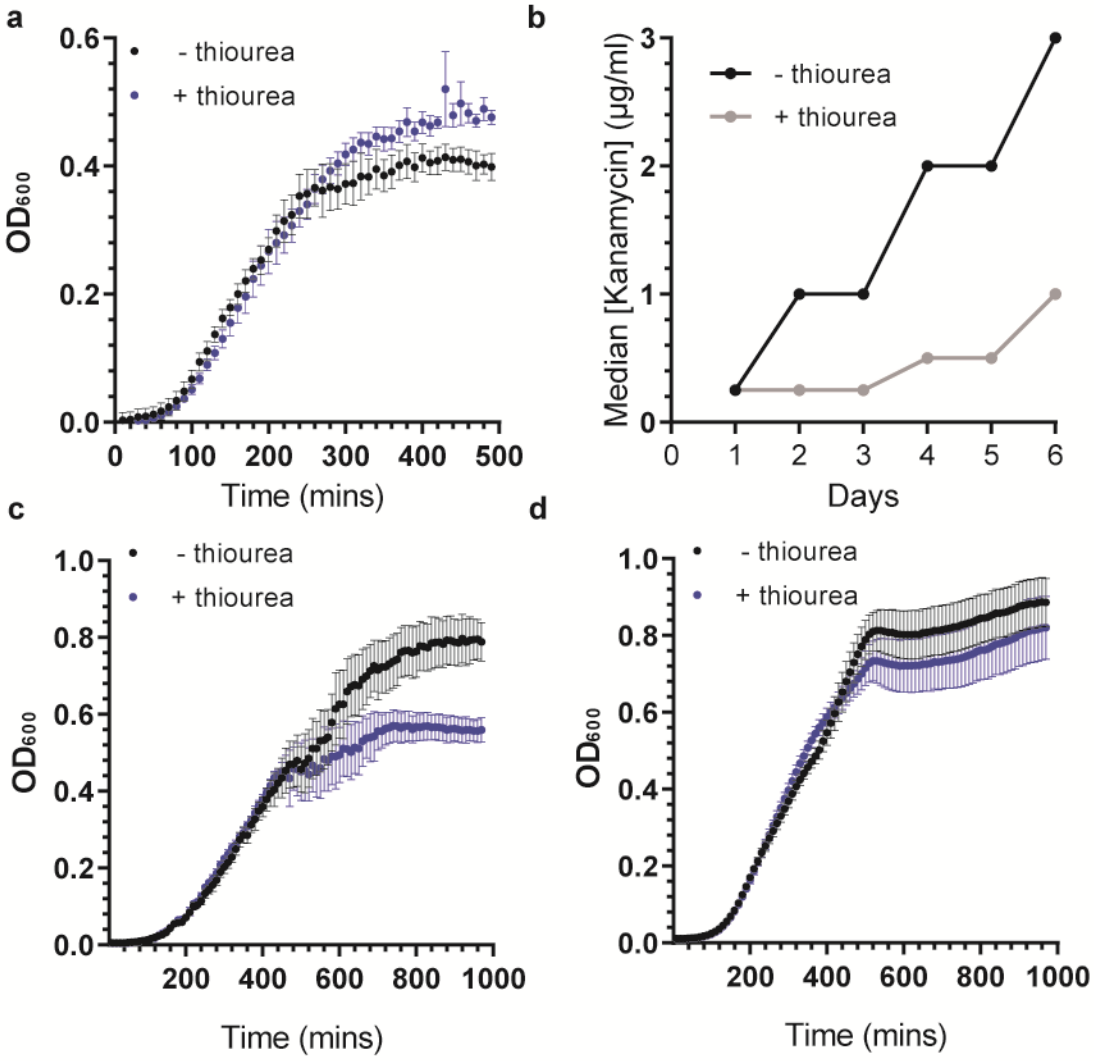
		Bottom	GTGGAACGCTA[dU]ATGTGCCATATAGCA GCGCGCCATTTCCACCAGAAT
polA for cloning into pET28a	*B. subtilis*	Fwd	AAGGATCCACGGAACGAAAAAAATTAGT GCTTGTAGAC
		Rev	AAGAATTCTTATTTCGCATCGTACCAAGA TGGGC
katA for cloning into pCAL838	*B. subtilis*	Fwd	TTAAGCTTATGAGTTCAAATAAACTGACA ACTAGCTGGG
		Rev	TTGCTAGCTTAAGAATCTTTTTTAATCGGC AATCCAAGGC

Extended Data Figure 1:



a) SDS-PAGE of purified *B. subtilis* PolA b) Primer extension assay with purified *B. subtilis* PolA using a gap substrate c) Mutation rates of Bacillus subtilis strains of the indicated genotype to rifampicin. n=40 (*ΔpolY1 ΔpolY2 ΔuvrA*), 40 (*ΔpolY1 ΔpolY2 ΔuvrB*), 30 (*ΔpolY1 ΔpolY2 ΔuvrC*), 33 (*ΔpolY1 ΔpolY2 Δmfd*), 36 (*ΔpolA ΔpolY1 ΔpolY2 ΔuvrA*), 36 (*ΔpolA ΔpolY1 ΔpolY2* Δmfd) biological replicates. Error bars are 95% confidence intervals. d) Primer extension assay with purified *B. subtilis* PolA using a substrate including an abasic site.

547 Extended Data Figure 2



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549 a) OD600 measured every 10 mins for 490 mins of wt *B. subtilis* with and without 50 mM
550 thiourea in the media. b) Median kanamycin concentration that allows for growth in wt cells at
551 each sampled timepoint. 50 mM thiourea was included in the media when indicated. n=12 (-
552 thiourea), 12 (+ thiourea) c,d) OD600 measured every 10 mins for 970 mins of *P. aeruginosa* (b)
553 and *S. aureus* (c) with and without 10 mM thiourea (*P. aeruginosa*) or 50 mM thiourea (*S.*
554 *aureus*) in the media. n= 12 biological replicates for all three strains. Error bars indicate standard
555 deviation.