Oxidation of dCTP contributes to antibiotic lethality in stationary-phase mycobacteria

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Growing evidence shows that generation of reactive oxygen species (ROS) derived from antibiotic-induced metabolic perturbation contribute to antibiotic lethality. However, our knowledge of the mechanisms by which antibiotic-induced oxidative stress actually kills cells remains elusive. Here, we show that oxidation of dCTP underlies ROS-mediated antibiotic lethality via induction of DNA double-strand breaks (DSBs). Deletion of *mazG*-encoded 5-OH-dCTP–specific pyrophosphohydrolase potentiates antibiotic killing of stationary-phase mycobacteria, but did not affect antibiotic efficacy in exponentially growing cultures. Critically, the effect of *mazG* deletion on potentiating antibiotic killing is associated with antibiotic-induced ROS and accumulation of 5-OH-dCTP. Independent lines of evidence presented here indicate that the increased level of DSBs observed in the *ΔmazG* mutant is a dead-end event accounting for enhanced antibiotic killing. Moreover, we provided genetic evidence that 5-OH-dCTP is incorporated into genomic DNA via error-prone DNA polymerase DnaE2 and repair of 5-OH-dC lesions via the endonuclease Nth leads to the generation of lethal DSBs. This work provides a mechanistic view of ROS-mediated antibiotic lethality in stationary phase and may have broad implications not only with respect to antibiotic lethality but also to the mechanism of stress-induced mutagenesis in bacteria.

DNA double-strand breaks | 5-OH-dCTP | reactive oxygen species | antibiotic | *Mycobacterium*

Antibiotics that inhibit essential biological processes can kill cells. However, recent studies demonstrated that, in addition to the well-studied mechanisms of killing through corrupting the function of primary targets, antibiotic-induced metabolic perturbations also have strong impact on antibiotic efficacy (1–3). For example, recent studies showed that generation of reactive oxygen species (ROS) as a consequence of metabolic perturbation contributed substantially to cell death by bactericidal antibiotics or other lethal stress (4–9). Despite a great deal of studies having established the link between ROS and antibiotic killing, the molecular mechanisms through which antibiotic-induced ROS actually kills cells remain largely unresolved.

Given that the antibiotic-induced ROS level within bacterial cells is far less than the levels observed in bacteria approaching lethality, it was perceived that oxidative damages to DNA are likely responsible for ROS-mediated cell death (10). In line with this, recent studies using *Mycobacterium tuberculosis* (*Mtb*, the causative agent of tuberculosis) and *Escherichia coli* showed that lethal doses of bactericidal antibiotics can induce double-strand breaks (DSBs) in a ROS-dependent manner (4, 11–15). In addition, ROS-dependent mutagenesis was also observed in bacteria exposed to sublethal doses of antibiotics (16, 17). Importantly, inactivation of the DSB-repair pathways through deletion of *recA* or *recB* substantially potentiates cell death by bactericidal antibiotics, suggesting DSB is a causal contributor to antibiotic lethality (10, 12, 14, 18).

Typically, ROS can directly induce DNA strand breaks via reaction with the sugar-phosphate backbone within the double helix. In addition, ROS also react with the DNA base moiety of incorporated or free nucleotides, leading to DSB generation during the DNA repair process (19). Because ROS-associated damage requires Fe^{2+}-mediated Fenton reaction, the pool of nucleotides may be subject to damage due to chelation of Fe^{2+} by triphosphates (20). This notion was supported by recent findings showing that oxidized dGTP (8-oxo-dGTP) substantially contributes to antibiotic-induced cell death and mutagenesis in *E. coli* (4, 10, 14, 16). Specifically, the critical contribution of 8-oxo-dGTP to antibiotic lethality relies on ROS generation, DNA polymerase III/IV/V's incorporation of 8-oxo-dGTP into DNA, and DSBs generated by incomplete base excision repair (9, 12, 14, 16). However, it remains unclear whether oxidized nucleotides other than 8-oxo-dGTP also contribute to antibiotic-induced DNA damage.

Our previous studies found that mycobacterial *mazG* encodes a 5-OH-dCTP–specific (an oxidized form of dCTP) pyrophosphohydrolase, with a K_m value of 1.9 μM (21, 22). Deletion of *mazG* in

Significance

Recent studies revealed that oxidative DNA damage is a common event downstream of the interaction of antibiotics with their molecular targets. For instance, exposure to lethal doses of antibiotics may cause double-strand breaks (DSBs), whereas exposure to sublethal doses of antibiotics may lead to mutagenesis. Therefore, detailed understanding of mechanisms underlying antibiotic-induced DNA damage may broaden our knowledge not only of antibiotic lethality but also the mechanism of stress-induced mutagenesis in bacteria. Here, we show that oxidation of dCTP underlies a mechanism of antibiotic-induced DNA damage and cell death. We demonstrate that antibiotic lethality stemming from 5-OH-dCTP relies on reactive oxygen species production, mispairing induced by DnaE2, and unrepaired DSBs generated by incomplete base excision repair via Nth.

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mycobacteria resulted in a 20-fold increase in the frequency of genomic CG-TA mutation both under oxidative stress and in the stationary phase of growth and led to virulence attenuation during persistent infection of *Mtb* in a mouse model (21). In the present study, we demonstrated that oxidized dCTP contributes to antibiotic lethality in stationary-phase mycobacteria. We provided a model in which 5-OH-dCTP could be incorporated into genomic DNA via error-prone DNA polymerase DnaE2, and in which incomplete repair of 5-OH-dC lesions via endonuclease Nth led to the production of lethal DSBs. Since lethality stemming from 5-OH-dCTP is only relevant in stationary phase, a multistress and nongrowing physiological state that is known to be associated with drug tolerance and mutagenesis, our finding may have broad implications not only for antibiotic lethality but also for the mechanism of stress-induced mutagenesis in bacteria.

Results

Deletion of *mazG* Results in Increased Antibiotic Killing of Stationary-Phase Mycobacteria and Intracellular *Mtb*. Deletion of *mazG* in *Mtb* or *Mycobacterium smegmatis* (*Msm*) did not affect bacterial growth in vitro and the minimal inhibitory concentration (MIC) of antituberculosis drugs (*SI Appendix*, Fig. S1 and Table S1). Besides, deletion of *mazG* in *Mtb* did not affect antibiotic killing of exponential cultures exposed to 100× MIC of isoniazid (INH, inhibits cell wall synthesis), rifampicin (Rif, inhibits transcription), streptomycin (Str, inhibits translation), and ciprofloxacin (Cfx, inhibits DNA synthesis) (*SI Appendix*, Fig. S2). However, in the stationary phase, the Δ*mazG* mutant yielded 10- to 300-fold fewer survivors than that of wild type after 8-d treatment with Rif, Str, or Cfx (Fig. 1 *A*–*C*). In addition, both the wild type and the Δ*mazG* mutant were equally refractory to the killing by INH (Fig. 1*D*), consistent with the fact that INH is ineffective to nongrowing mycobacteria (23). In accordance with its role in stationary phase, transcription of *mazG* was increased about 5-fold over that in exponential growth phase (*SI Appendix*, Fig. S3). Transformation of the Δ*mazG* mutant with an integrative plasmid encoding a wild-type *mazG* allele fully restored the survival defect (Fig. 1 *A*–*C*).

To assess whether deletion of *mazG* potentiates antibiotic killing of *Mtb* during infection of macrophages, *Mtb*-infected macrophages were treated with antibiotics. *mazG* deletion caused attenuated intracellular survival (without antibiotic treatment) after 2 d postinfection of human-derived THP-1 macrophages (*SI Appendix*, Fig. S4), consistent with our previous results using RAW264.7 macrophages (21). After 1 d postinfection, infected macrophages were exposed to antibiotics at a concentration of 10× MIC. The results showed that the survival ratio (compared with untreated control) of the Δ*mazG* mutant is three- to five-fold lower than that of wild type and the complemented mutant after 2-d exposure to Rif or Str, respectively (Fig. 1*E*).

Similar to the results using *Mtb*, deletion of *mazG* in *Msm* also resulted in 10- to 1,000-fold decreases of survival compared with wild type after exposure of stationary cultures to Rif or Cfx for 12 d or Str for 12 h (Fig. 1 *F*–*H*). Because the MazG is biochemically and functionally conserved between *Mtb* and *Msm* (21, 22), we used *Msm* as a model system for mechanistic studies in the following sections.

Increased Antibiotic Killing in the Δ*mazG* Mutant Can Be Attributed to 5-OH-dCTP. If the increased antibiotic killing observed in the Δ*mazG* mutant is caused by accumulation of 5-OH-dCTP, exogenous 5-OH-dCTP may induce a similar effect in wild type. It was known that extracellular pyrimidines could affect the intracellular concentration of pyrimidine nucleotides in *Msm*, presumably via the transport by nucleoside cation symporter (MSMEG_5730 and MSMEG_0564) (24, 25). We observed that addition of 100 μM 5-OH-dCTP or dCTP to culture media did not affect bacterial growth, consistent with previous results on *Msm* and *E. coli* (21, 26). As shown in Fig. 2 *A*–*C*, exponential cultures of the wild-type *Msm* grown in the presence of 100 μM 5-OH-dCTP became more susceptible to killing by Rif and Cfx than that of untreated, while no effect was observed by Str treatment. As a control, the supplement of dCTP did not potentiate killing by Rif and Str, but induced a modest increase of killing by Cfx compared with that of untreated, indicating that imbalance on cellular dNTP pool caused by exogenous dCTP may lead to lethal DNA damage when DNA synthesis is interrupted by Cfx (27). Intriguingly, the combination of dCTP and Rif/Str did not potentiate cell death, suggesting that the increased intracellular dCTP pool does not lead to accumulation of 5-OH-dCTP or that wild-type cells are able to handle this slight elevation in 5-OH-dCTP produced from added dCTP. This speculation is supported by the recent study showing that the pronounced mutational spectra of the *dcd* mutant (3.7-fold increase of intracellular dCTP) were not derived from oxidative damage to cytosine (28).

Moreover, a similar effect of exogenous 5-OH-dCTP on potentiating antibiotic killing was observed in starvation-induced nongrowing *Msm* (Loebel model) (29) (Fig. 2 *D* and *E*). The exception is that supplementation of either 5-OH-dCTP or dCTP resulted in slightly reduced survival during the course of nutrient starvation (Fig. 2*D*). This reduced survival might be a result of the imbalance of the dNTP pool created by the exogenous supplementation of dCTP, be it oxidized or not. Such dNTP imbalance is known to be cytotoxic to nongrowing cells due to low rate of dNTP turnover and diminished DNA repair activities (30–32). Together, these results suggest that 5-OH-dCTP could elicit lethal effect upon antibiotic exposure (3).

Increased Antibiotic Killing Caused by *mazG* Deletion Is Associated with ROS. Next, we thought to determine the mechanism of how the *mazG* depletion potentiates cell death by antibiotics. We first

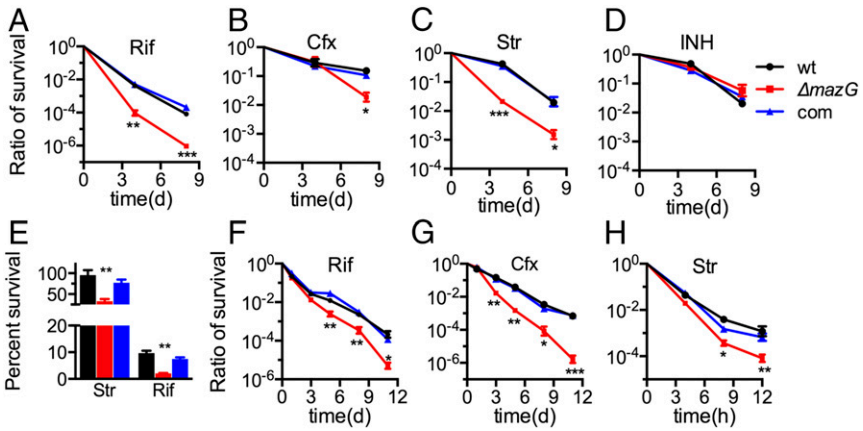

Fig. 1. Deletion of *mazG* results in increased antibiotic killing. (*A*–*D*) Survival of stationary-phase cultures of *Mtb* strains treated with 10 mg/L Rif (*A*), 20 mg/L Cfx (*B*), 10 mg/L Str (*C*), or 10 mg/L INH (*D*). (*E*) After infection of THP-1 macrophages for 1 d, *Mtb*-infected macrophages were exposed to 1 mg/L Rif, 4 mg/L Str, or left untreated for 2 d. (*F*–*H*) Survival of stationary-phase cultures of *Msm* strains treated with 200 mg/L Rif (*F*), 30 mg/L Cfx (*G*), and 10 mg/L Str (*H*). com, the complemented mutant; wt, wild type. In all panels, survival was determined by monitoring colony-forming units (cfu) and was expressed relative to untreated control. Data shown are mean ± SE in triplicate. Significant values are $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

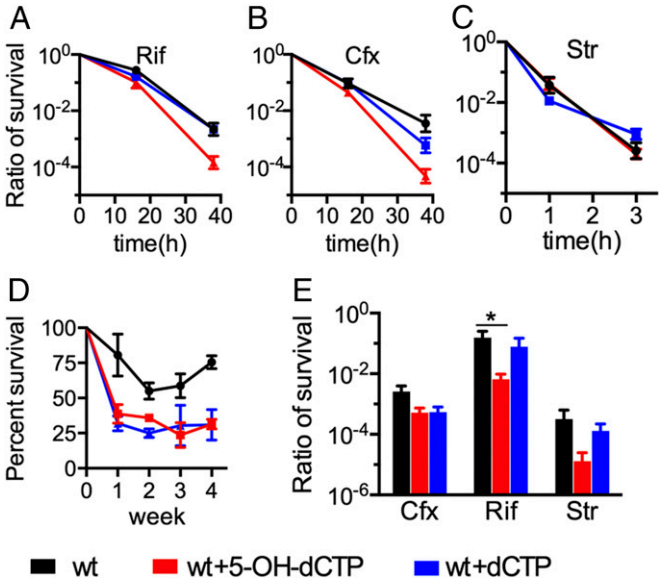

Fig. 2. Exogenous 5-OH-dCTP potentiates antibiotic killing of wild-type *Msm*. (*A*–*C*) Survival of exponential-phase *Msm* grown with the supplement of 100 μM nucleotides after exposure to 200 mg/L Rif (*A*), 30 mg/L Cfx (*B*), and 10 mg/L Str (*C*). (*D*) Survival of *Msm* during nutrient starvation (as described in *SI Appendix, SI Materials and Methods*) with the supplement of dCTP or 5-OH-dCTP. (*E*) Bacilli starved for 3 wk were treated with Rif or Cfx for 5 d, and with Str for 8 h. In all panels, bacterial survival was determined by monitoring cfus and expressed as the ratio compared with untreated control. Data shown are mean ± SE with at least three independent repeats. Significant values are $*P \leq 0.05$.

assessed whether antibiotic treatment of stationary cultures could induce ROS, which may contribute to nucleotide oxidation and antibiotic killing (13, 33). Using fluorescent dye dihydroethidium (a probe for superoxide) and flow cytometry (11, 13), we found that the ROS levels were significantly elevated during the course of treatment with Rif, Cfx, and Str compared with the untreated control, whereas no significant difference was observed between wild-type *Msm* and the Δ*mazG* mutant (Fig. 3*A*). In accordance with these results, qRT-PCR analyses showed that expression of *aphC* and *katG,* two functionally characterized genes responsive to oxidative stress in *Mycobacterium* (5), increased 2- to 5-fold in wild type and the Δ*mazG* mutant after exposure to Cfx for 24 h and Str for 90 min (treatment durations were chosen such that the survival ratio reduced about 1-log compared with that of the untreated) (Fig. 3*B*). In addition, we found that expression of *recA* was increased ∼40-fold and ∼5-fold upon Cfx and Str treatment, respectively. Meanwhile, expression of *dnaE2* was also increased ∼5-fold upon Cfx treatment, while no change was observed upon Str treatment (34). Notably, our results showed that induction of *recA* by antibiotics was more pronounced than that by 5 mM H_2O_2 (Fig. 3*B*), implying that intrinsically induced ROS by antibiotics is potent in causing DNA damage in stationary phase. Together, these results suggest that exposure to antibiotics leads to ROS production and DNA damage in stationary-phase *Msm* (34).

If the increased antibiotic killing caused by *mazG* deletion is indeed associated with ROS, exposure of the Δ*mazG* mutant to oxidant may result in a similar effect as that of antibiotic treatment. As expected, we found that the stationary-phase culture of the Δ*mazG* mutant was more susceptible to killing by 1 mM tert-butyl hydroperoxide (t-BHP, a stable oxidant) (35), showing a 10-fold decrease of survival compared with that of wild type after treatment for 1 d. Exposure of exponential cultures of the Δ*mazG* mutant to t-BHP also resulted in increased killing over that of wild type, although to a lesser extent than that of the stationary cultures (Fig. 3*C*).

MazG Depletion Leads to Increased DSB upon Exposure to Antibiotics. Because *mazG* deletion results in a mutator phenotype in stationary phase (21), we speculate that DNA damage induced by 5-OH-dCTP is likely the cause for increased antibiotic killing of the Δ*mazG* mutant (14, 21). To address this, we measured the DSB levels by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. As shown in Fig. 3*D*, while the DSB levels were slightly increased or unchanged in wild type and the complemented mutant during antibiotics treatment, it was substantially elevated in the Δ*mazG* mutant, showing a sevenfold increase after treatment with Rif and Cfx for 7 d or Str for 4 h compared with the untreated controls. These results indicate that the increased killing of the Δ*mazG* mutant by antibiotics correlates with DSB generation.

Increased DSB in the *ΔmazG* Mutant Causally Contributes to Increased Antibiotic Killing. To confirm that the elevated DSB level in the Δ*mazG* mutant is a dead-end event of antibiotic killing, we first assessed the susceptibility of DSB-repair deficient mutants to antibiotic killings. *Mycobacterium* species encode three DSB repair pathways, including homologous recombination (HR), nonhomologous end-joining (NHEJ), and single-strand annealing (SSA) (36). Deletion of *recA* (HR^-), *ligD* ($NHEJ^-$), or *recD* (SSA^-) in *Msm* did not affect bacterial growth in vitro (*SI Appendix*, Figs. S5 and S6). We found that both the HR^- strain and the $NHEJ^-$ strain showed increased antibiotic killing regardless of growth stages, whereas the SSA^- strain showed no such effect on antibiotic killing (Fig. 4*A* and *SI Appendix*, Fig. S6). Of note, our results demonstrated that the HR pathway plays a pronounced role in impairing antibiotic killing in stationary-phase *Msm* (Fig. 4*A*). Consistent with previous studies in *E. coli* (4, 14, 18), these results suggested that unrepaired DSB contributes to antibiotic killing of *Msm*.

We reasoned that if the increased DSB is a dead-end event in the Δ*mazG* mutant, inhibition of HR-mediated DSB repair would

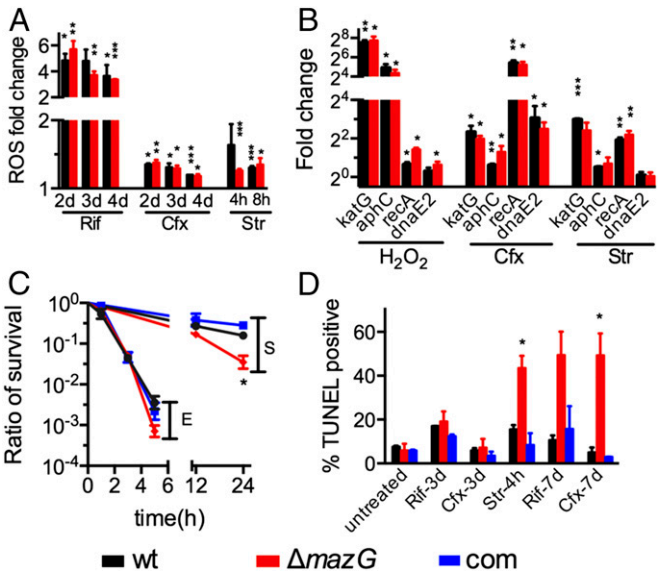

Fig. 3. Increased antibiotic killing caused by *mazG* deletion is associated with ROS and DSB. Stationary cultures of *Msm* strains were exposed to 200 mg/L Rif, 30 mg/L Cfx, or 10 mg/L Str. (*A*) Intracellular ROS levels measured by fluorescent dye dihydroethidium and flow cytometry at indicated times after exposure to antibiotics. (*B*) qRT-PCR analysis of gene expression after exposure to Cfx for 24 h, Str for 90 min, and 5 mM H_2O_2 for 30 min. (*C*) Survival of exponential-phase (E) and stationary-phase (S) cultures after exposure to 1 mM tert-butyl hydroperoxide (a stable oxidant). (*D*) DSB levels measured by TUNEL assay at indicated time points. com, the complemented mutant; wt, wild type. Data shown are mean ± SE in triplicate. Significant values are $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

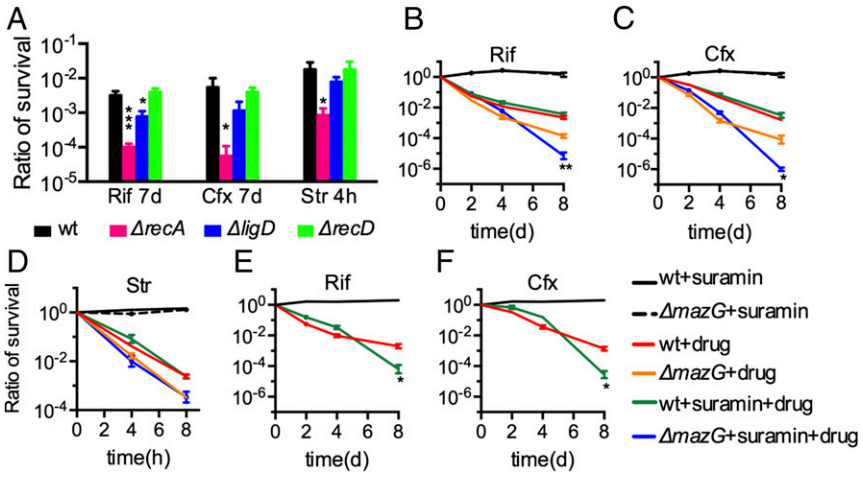

Fig. 4. Formation of DSB contributes to antibiotic lethality in *Msm*. (*A*) Survival of stationary cultures of *Msm* wild type (wt) and the DSB repair-deficient mutants after exposure to 200 mg/L Rif, 30 mg/L Cfx, or 10 mg/L Str at the indicated times posttreatment. (*B–D*) Survival of stationary-phase cultures of *Msm* wild type (wt) and the Δ*mazG* mutant after exposure to 200 mg/L rifampicin (*B*), 30 mg/L Cfx (*C*), and 10 mg/L Str (*D*) with or without the supplement of 50 μM suramin. (*E* and *F*) Same as *B* and *C* except that suramin was supplemented at 500 μM. Data shown are mean ± SE in triplicate. Significant values are $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

further increase antibiotic killing in the mutant. Suramin is an inhibitor of DNA strand exchange and ATPase activities of mycobacterial RecA (37). We found that addition of suramin alone (50–500 μM) did not affect viability of stationary-phase *Msm* and the Δ*mazG* mutant (Fig. 4 *B–F*). When combined with antibiotics, suramin supplemented at high concentration (500 μM) potentiated killing by Rif and Cfx upon stationary-phase wild-type *Msm* (Fig. 4 *E* and *F*), while no effect was observed at low concentration (50 μM), which is likely caused by the low efficacy of transmembrane transport of suramin (37). However, addition of 50 μM suramin resulted in a 20-fold increase of killing by Rif and Cfx in the Δ*mazG* mutant after treatment for 8 d (Fig. 4 *B* and *C*), suggesting that maintenance of full activity of DSB repair function is crucial for the Δ*mazG* mutant during antibiotic treatment. Of note, suramin effect is RecA dependent, as the addition of suramin did not potentiate antibiotic killing of the Δ*recA* mutant (*SI Appendix*, Fig. S7). Suramin did not affect the killing of all tested strains by Str (Fig. 4*D*), which is likely due to the inhibition of protein translation by Str overwhelming the inhibitory effect of suramin on RecA. Together, these results demonstrate that the increased DSB is a causal factor for increased antibiotic killing of the Δ*mazG* mutant.

Oxidized dCTP Is Incorporated into DNA via Error-Prone DNA Polymerase DnaE2. Based on the evidence that 5-OH-dCTP could be incorporated into DNA via the DNA polymerase lacking proofreading activity (38) and that error-prone DNA polymerase is the dominant polymerase for DNA synthesis in stationary-phase bacteria (32, 39), we hypothesized that mycobacterial *dnaE2*, the functional homolog of *dinB*, might be contributing to the increased antibiotic killing in the Δ*mazG* mutant (34). As expected, expression of *dnaE2* was increased 40-fold in stationary-phase cultures over that in exponential phase (*SI Appendix*, Fig. S8) (32, 39). Deletion of *dnaE2* alone (*SI Appendix*, Fig. S5) did not affect antibiotic sensitivity in stationary-phase *Msm*. However, inactivation of *dnaE2* in the Δ*mazG* background (Δ*mazG*Δ*dnaE2*) led to a striking reduction in antibiotic killing (Fig. 5). In addition, deletion of *dnaE2* in the Δ*mazG* mutant only partially rescued the survival defect of the Δ*mazG* mutant exposed to Cfx (Fig. 5*B*), indicating that Cfx may also cause DNA damage in a DnaE2-independent manner, which is consistent with the model of Cfx's action (18).

Interestingly, although *dnaE2* deletion did not affect antibiotic efficacy in killing of stationary-phase cultures, it reduced killing of exponential-phase *Msm* by Rif and Cfx compared with that of wild type, but did not affect susceptibility to Str (*SI Appendix*, Fig. S9). This finding is consistent with previous observations of *dinB*'s contribution to antibiotic killing of exponentially growing *E. coli* (12, 14).

DSB Induced by 5-OH-dCTP Is Generated During DNA Repair via Nth. Previous studies showed that antibiotic-induced DSB occurs during incomplete base excision repair of DNA lesions (10, 12, 14, 32, 39). If so, deletion of the 5-OH-dC repair endonuclease *nth* in the Δ*mazG* background may restore antibiotic killing (40). We therefore constructed and tested a Δ*nth*Δ*mazG* mutant (*SI Appendix*, Fig. S5) and observed a striking reduction in antibiotic killing in the mutant, resulting in a survival ratio comparable to that of wild-type *Msm* (Fig. 6*A*). Importantly, we found that deletion of *nth* in the Δ*mazG* mutant substantially reduced the DSB to the wild-type level (Fig. 6*B*), demonstrating that 5-OH-dCTP–induced DSB is generated during the processing of 5-OH-dC lesions by Nth. As expected, deletion of *nth* in the Δ*mazG* mutant resulted in reduced repair of DNA damage, leading to a threefold increase of rifampicin-resistance mutation frequency compared with that of the Δ*mazG* mutant during stationary phase (Fig. 6*C*). However, the mutator effect of the Δ*nth*Δ*mazG* mutant did not affect the efficacy of antibiotic killing, as the proportion of antibiotic-resistant mutants remained unchanged during the course of antibiotics treatment (note that stationary phase is a growth-limited environment) (*SI Appendix*, Fig. S10). Intriguingly, deletion of *nth* alone in *Msm* (Δ*nth*) also results in increased antibiotic killing albeit to a lesser extent than that in the Δ*mazG* mutant (Fig. 6*A*), suggesting that unrepaired oxidized pyrimidine lesions could also affect genome stability via a different mechanism, e.g., replication fork block (41).

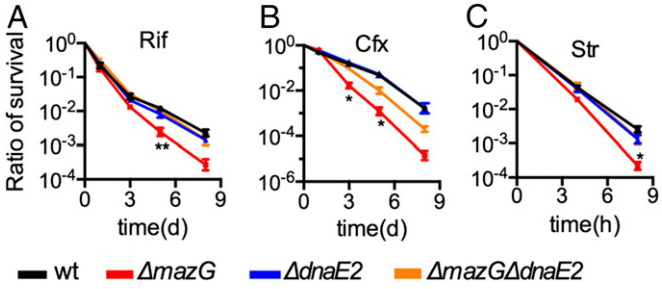

Fig. 5. Deletion of *dnaE2* reduces antibiotic killing of the Δ*mazG* mutant. Stationary-phase *Msm* cultures were exposed to 200 mg/L Rif (*A*), 30 mg/L Cfx (*B*), or 10 mg/L Str (*C*). Bacterial survival was determined by monitoring cfus and expressed relative to cfus before treatment. wt, wild type. Data shown are mean ± SE in triplicate. Significant values are $*P \leq 0.05$, $**P \leq 0.01$.

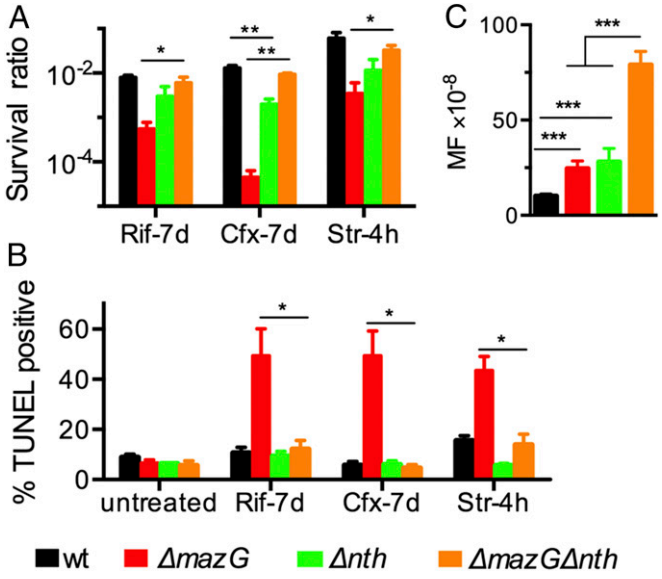

Fig. 6. DSB induced by 5-OH-dCTP is generated during DNA repair via Nth. (*A*) Survival of stationary-phase cultures after exposure to 200 mg/L Rif, 30 mg/L Cfx, or 10 mg/L Str at the indicated times posttreatment. (*B*) DSB levels measured by TUNEL assay at the indicated times posttreatment. (*C*) Rif-resistant mutation frequencies (MFs) of stationary-phase *Msm* strains. Data shown are mean ± SE with at least three independent experiments. Significant values are $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

Discussion

Despite increasing evidence suggesting the strong impact of ROS on antibiotic lethality, experimental evidence addressing the actual mechanism through which antibiotic-induced ROS kills cells remains limited. This knowledge gap makes the conclusion of antibiotic-induced ROS contributing to cell death confusing, especially when considering the fact that antibiotic-induced ROS level within bacterial cells is far less than the levels seen in bacteria approaching lethality. In this study, we revealed that oxidation of dCTP substantially contributes to antibiotic-induced DNA damage and cell death in stationary-phase mycobacteria. Overall, our results provided a model that antibiotic lethality stemming from 5-OH-dCTP relies on ROS production, DnaE2's incorporation of 5-OH-dCTP, and unrepaired DSB generated by incomplete base excision repair via Nth. These findings, taken together with the previous studies showing that oxidation of dGTP contributes to antibiotic lethality (4, 9, 14, 16), indicate that oxidative damage of nucleotides underlies a major mechanism of antibiotic-induced DSB and cell death (1–3).

It remains unclear whether oxidized dGTP is associated with antibiotic lethality in *Mycobacterium*. However, the observations of lack of genomic AT-CG mutation (induced by incorporation of 8-oxo-dGTP) during *Mtb* infection suggest that *Mycobacterium* is potent for sanitizing 8-oxo-dGTP (42). In this connection, it is worth noting that *Mycobacterium* encodes four MutT proteins (43).

The increased antibiotic killing in the Δ*mazG* mutant is only relevant in stationary phase, consistent with our previous results that 5-OH-dCTP–mediated mutagenesis prominently occurred in stress conditions (21). It has long been known that nongrowing bacilli are tolerant to antibiotic killing. For example, single-cell studies using either *Msm* or *E. coli* showed that cells that survive transient antibiotic treatment were enriched in a nongrowing subpopulation compared with their fast-growing kin (44, 45). Importantly, recent studies showed that the mounting of host immune responses could stimulate the formation of nongrowing *Mtb*, demonstrating that drug tolerance has been implicated in persistent infections (46, 47). A prevailing view suggested that diminished requirement of antibiotic target in nongrowing bacilli contributes to drug tolerance (23, 44, 48). However, our results indicate that tolerance to antibiotics is not engendered solely by reduced antibiotic target corruption. Rather our findings demonstrate that active prevention of antibiotic-induced damages as an intrinsical response in stationary-phase bacteria contributes to antibiotic tolerance. Recent studies provided other evidence supporting this theory, demonstrating that antioxidant responses and DSB-repair functions contributed to antibiotic tolerance (18, 49).

The causes of this growth stage-dependent effect of 5-OH-dCTP on antibiotic lethality remain to be elucidated. It was known that mechanisms of DNA synthesis and repair are different between these two growth stages (32, 39). For instance, differential expression of *dnaE2* between these two stages (*SI Appendix*, Fig. S8) may affect the rate of DNA synthesis, mutagenesis, and the consequence of DNA damage (see also discussion below). Since the lethality stemming from oxidized nucleotides relies on multiple molecular events, differential regulation of these molecular events between exponential phase and stationary phase may synergistically affect the phenotype readouts (9, 14, 16). This notion is supported by the observations that the effect of *dnaE2* deletion on antibiotic killing differed between exponential phase and stationary phase (Fig. 5 and *SI Appendix*, Fig. S9). In this connection, it is worth noting that formation of 5-OH-dCTP–induced DNA damage accumulated over time in stationary-phase *Msm*, according to the following two observations: (*i*) the increased DSB level in the Δ*mazG* mutant was observed only in the late stage of drug exposure (Fig. 3*D*), and (*ii*) suramin's effect did not begin until after day 4 in stationary phase (Fig. 4 *B*–*F*).

The increased sensitivity of the *mazG* mutant to antibiotic killing can be substantially suppressed by deletion of *dnaE2* (Fig. 5), suggesting that DnaE2 is a major factor mediating incorporation of 5-OH-dCTP. In *E. coli*, however, 8-oxo-dGTP–induced DNA damage upon antibiotic treatment is mediated by *dinB* (DNA Pol IV), *umuDC* (DNA Pol V), and *dnaE* (essential replication DNA polymerase Pol III)'s incorporation into genomic DNA (14). Because both *dinB* and *umuDC* belong to the SOS regulon that could be induced by antibiotic treatment (6), it is likely that Pol III contributes largely to the incorporation of 8-oxo-dGTP into DNA in *E. coli* under exponential growth phase. This speculation is supported by the biochemical evidence that Pol III indeed incorporate 8-oxo-dGTP and by the genetic evidence that deletion of *mutT* results in a strong mutator phenotype under exponential phase (50). These results suggest that the differential mechanisms of incorporation of oxidized nucleotides may determine the phenotype readouts.

Consistent with previous findings of *dinB* (Pol IV) overexpression in *E. coli* (14), the results of *dnaE2* deletion (Fig. 5 and *SI Appendix*, Fig. S9) demonstrate that the incorporation of oxidized dNTP by error-prone polymerase is deleterious for bacteria exposed to lethal doses of antibiotics. This model of action appears counterintuitive when considering this translesion DNA polymerase could enable bacterial survival of UV-induced DNA damage by allowing bypass of lethal replication-blocking lesions (34). However, the deleterious role of DinB/DnaE2 in potentiating antibiotic killing can be understood by recognizing that Pol IV can also function as an alternative replisome, especially under stress conditions when the rate of DNA replication is reduced (31, 32, 51). Under this circumstance, DinB/DnaE2 may promote mispairing and thus, lead to lethal DSB during the process of DNA repair.

Our work exemplifies how antibiotic-induced ROS can actually contribute to DNA damage and cell death. However, it is worth noting that oxidation of nucleotides can also promote mutagenesis under sublethal conditions (16, 17). For example, our previous study demonstrated that the rifampicin-resistance mutation frequency in the *mazG*-null mycobacteria was significantly increased both under oxidative stress and in the stationary

phase of growth (21). Therefore, this paradoxical aspect of DNA damage on antibiotic efficacy should be considered when exploiting these damage-preventing pathways for the development of new therapeutic strategies and antimicrobial adjuvants.

Materials and Methods

Detailed materials and methods can be found in *SI Appendix, SI Materials and Methods*. Mycobacterial strains and plasmids, culture conditions and drug treatment, nutrient starvation, determination of rifampicin-resistance mutation frequency, TUNEL assay, ROS measurement, quantitative real-time PCR, macrophage infection, and statistical analysis are described therein. Mycobacterial strains and primers used are listed in *SI Appendix*, Tables S2 and S3.

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