Supplementary Materials for

Oxidation of the Guanine Nucleotide Pool Underlies Cell Death by Bactericidal Antibiotics

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Materials and Methods

Strains and plasmids

The strains used in this study are listed in Table S3. Alleles were transduced via standard P1*vir* transduction. All plasmids were from the ASKA over-expressing plasmid library (*38*) except for the DinB over-production plasmid. The DinB over-production plasmid (pINIII::DinB) was generated by ligating a DinB PCR product created using primers: 5′-GGGGGGTCTAGAATGCGTAAAAATCATTCATGTGG and 5′-CCCCCCCATATGTCATAATCCCAGCACCAGTTGTCTTTCC into the XbaI and NdeI sites of pINIII (*39*). The DinB F13V over-production plasmid pINIII::DinB F13V was generated using standard site-specific mutagenesis procedures.

DinB over-production growth conditions

For aerobic conditions, cells were freshly transformed with pINIII::DinB and used to inoculate 5 ml of LB 0.2% glucose with appropriate antibiotics for plasmid selection. Cultures were grown overnight without aeration at 37°C. The over-night cultures were diluted with 20 ml 1X M9 Salts and subsequently centrifuged at 7500 g for 5 minutes. The pellets were then resuspended in 20 ml M9 0.2% glucose media with antibiotics necessary for plasmid selection, then diluted another 20 fold. Cultures were grown at 37°C to an OD_{600} of 0.1 with heavy aeration at which time DinB was induced with 1 mM IPTG and if appropriate thiourea (100 mM final concentration; Sigma) or 2,2′-dipyridyl (500 µM final concentration; Sigma) final concentration. Aliquots were taken at the indicated time point for further analysis.

For anaerobic experiments, 2 ml of M9 glucose ampicillin was inoculated with freshly transformed pINIII::DinB colonies in a 2 ml screwcap vial. After 24 hours of growth at 37°C, IPTG was added (1 mM final) for further growth overnight. Cultures were then serially diluted and plated on LB ampicillin plates for further growth at 37°C in an anaerobic chamber (BD Biosciences) overnight.

Antibiotic sensitivity growth conditions

Strains were grown overnight without aeration at 37°C in LB and, if appropriate, antibiotics for plasmid selection. Overnight cultures were diluted 1:100 in fresh LB media or LB chloramphenicol with 1mM IPTG and grown at 37°C to an OD_{600} of 0.1. For most experiments, antibiotics were added at a final concentration of 5 µg/ml ampicillin, 250 ng/ml norfloxacin, 5 µg/ml kanamycin, and 40 µg/ml penicillin (Sigma). For Δ*rec*A and Δ*rec*B experiments, antibiotics were added at 2 µg/ml ampicillin, 25 ng/ml norfloxacin, and 3 µg/ml kanamycin. After drug addition, aliquots were taken at the indicated time point for further analysis.

Primer extension

Primer template DNA substrates with A, G, C, and T at the templating position (Below) were purified and [γ-^{32}P] ATP (Promega) end-labeled as described previously (*40*). Wild-type DinB and DinB F13V were purified as previously described (*41*). The following assay components were incubated for 10 minutes at 25°C: 10 nM primer template DNA, 1 µM DinB or DinB F13V, in buffer consisted of 50 mM HEPES (pH 7.5), 100 mM KCl, 7.5 mM $MgCl_2$, 5% glycerol and 0.1% bovine serum albumin, prior to initiating the reaction with 500 µM of either dGTP (GE Healthcare) or 8-oxo-dGTP (TriLink). The reactions were quenched after 10 minutes

with 500 mM EDTA, products were separated on 20% denaturing polyacrylimide gels. Bands were quantified using a Typhoon Imager (GE Healthcare).

Primer template DNA substrates:

5′-TCGCAGCCGGTCCA
3′-AGCGTCGGCCAGGTXCCCAAA X = A or G or T

5′-TCGCAGCCGGTCCA
3′-AGCGTCGGCCAGGTCTCCAAA

RNA collection and microarray analysis

RNA was isolated from DinB over-producing cells using the QIAGEN RNeasy extraction kit and samples were treated with DNase using DNA-free (Ambion). cDNA preparation and microarray analysis were performed as previously described (*42*). The resulting microarray * .CEL files were combined with * .CEL files from arrays that comprise the M3D compendium (*43*) (http://m3d.bu.edu) and RMA normalized (*44*) with RMAexpress. Each gene's standard deviation of expression, σ, was calculated across the entire compendium and used to construct the z-scale difference between that gene's normalized expression following treatment versus the untreated control:

$$\Delta z_{\exp} = \frac{X_{\exp} - X_{\mathrm{ctl}}}{\sigma}$$

This allowed us to measure each gene's change in expression for a given experiment in units of standard deviation, a form of the z-test.

RibA Western

MG1655 cells over-producing His-RibA (pCA24N::His RibA) were treated with kanamycin for 15 to 60 minutes. Whole cell lysates were then prepared by resuspending cells in lysis buffer (50 mM Tris–HCl, pH 8.0, 2 mM EDTA, and 0.3 mg/ml lysozyme) and incubating on ice for 60 minutes. The lysates were cleared by centrifugation and 0.05 μg protein separated on a 4-20% polyacrylimide gel. Proteins were transferred to a PVDF membrane and probed for RibA-His content using anti-His antibody (1:2000; Qiagen) and anti-mouse IgG antibody conjugated to Alexa488 (1:10,000; Invitrogen). RibA-His bands were visualized and quantitated using a Typhoon Imager and Image Quant Software (GE Healthcare).

TUNEL

Approximately 10^6 cells were permeabilized by resuspension in 4% paraformaldehyde in PBS (Boston Bioproducts) and incubation on ice for 60 minutes. The cells were then washed twice in cold PBS, resuspended in 70% ethanol, and stored over-night at -20°C. Cells were then resuspended and washed with the wash buffer provided by the Apo-Direct Kit (BD Pharmingen). Labeling was performed by resuspending cells in labeling solution containing 1x reaction buffer, TdT enzyme, and FITC-dUTP from the Apo-Direct Kit for 60 minutes at 37°C. Cells were then diluted and washed with rinse buffer followed by resuspension in propidium iodine counter stain. Stained cells were then analyzed by microscopy and flow cytometry. Pseudocolor plots and cell histograms were generated in FlowJo software after gating out cells that did not stain for propidium iodine.

HPF dye measurements

Cells were grown overnight in LB media without shaking at 37°C. Cells were then resuspended in M9 minimal media and grown to an OD of 0.1 at 37°C with shaking at 300rpm. Cells were then induced with 1mM IPTG and grown for three hours at 37°C with shaking at 300 rpm. Samples were collected, centrifuged for 1 minute at 10,000 rpm, and resuspended in 1X PBS plus 5mM 3'-p-hydroxyphenyl fluorescein (HPF). Samples were then incubated at room temperature in the dark for 15 minutes, then centrifuged at 10,000 rpm and resuspended in PBS for FACS analysis.

8-oxo-GTP hydrolysis

Era hydrolysis of 8-oxo-GTP (TriLink) and GTP (Sigma) was measured using the BIOMOL Green™ Reagent Kit (Enzo Life Sciences). Reactions containing 5 μM Era in 50 mM Tris-HCl 200 mM NaCl and 5 mM $MgCl_2$ were initiated with 125 μM GTP or 8-oxo-GTP. After the indicated time, an aliquot was removed and added to the BioMol Green Reagent at a 1:2 reaction to reagent ratio and incubated for 30 minutes prior to OD_{620} measurements.

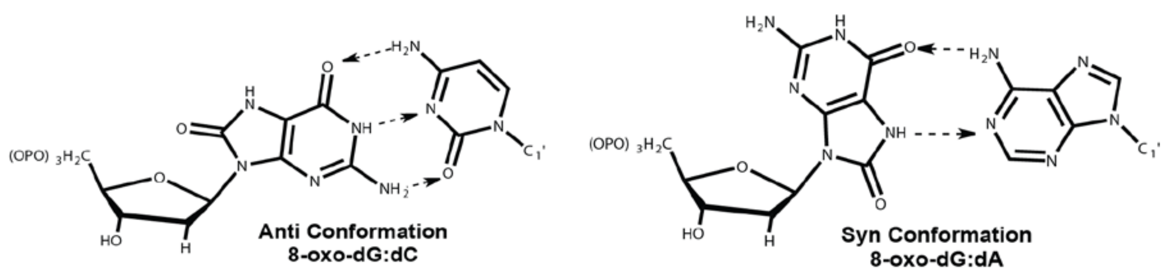

Fig. S1.
Potential mutagenesis associated with oxidation of guanine. 8-oxo-dG can basepair with a cytosine or adenine when in the anti- and syn- conformations, respectively.

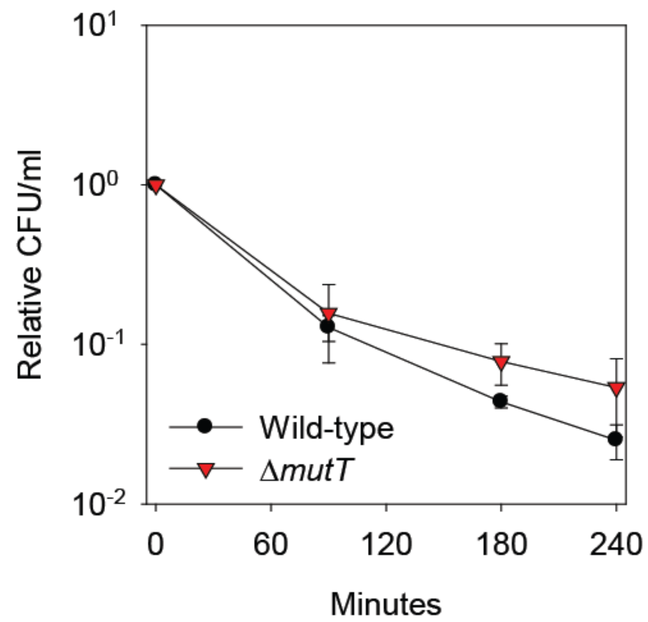

Fig. S2

Deletion of *mutT* does not sensitize cells to DinB over-production. DinB over-production in wild-type (black) and Δ*mutT* (red) cells results in similar cytotoxicity as measured by relative CFU/ml.

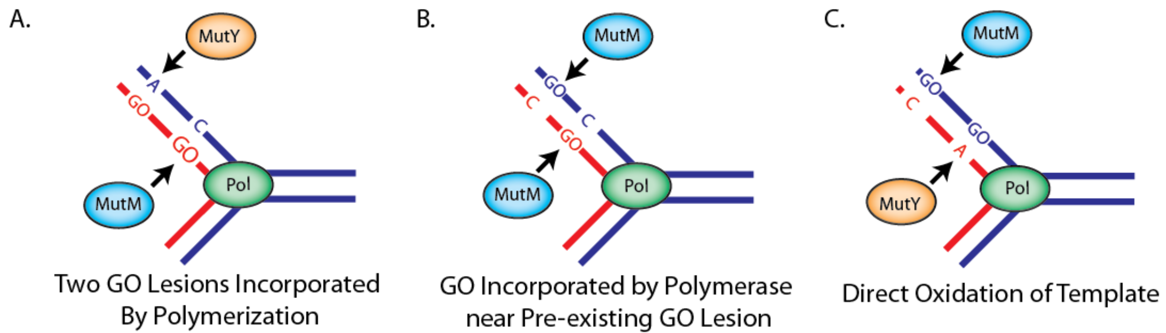

Fig. S3

Closely spaced 8-oxo-dG residues and subsequent action by MutM and MutY DNA glycosylases can potentially result in DSBs if base excision repair is initiated on opposite strands. A. Closely spaced 8-oxo-dG lesions can occur when they are incorporated into the daughter strand during DNA synthesis, either because the DNA polymerase involved is prone to using 8-oxo-dGTP as a substrate (i.e., DinB/DNA Pol IV) or because intracellular 8-oxo-dGTP levels are so high that 8-oxo-dG is incorporated by more stringent DNA polymerases (i.e., DnaE/DNA Pol III). A lethal DSB can be generated by the action of MutM and MutY on the daughter and template strands, respectively. B. If an 8-oxo-dG was introduced into the daughter strand across from a dC during replication near a GO in the template strand, a DSB could be formed by the subsequent action of MutM on both the template and daughter strand. C. The occurrence of a closely spaced dC:8-oxo-dG and 8-oxo-dG:C pair as a consequence of direct oxidation of DNA could also result in DSBs, but they would not be suppressible by over-production of MutT

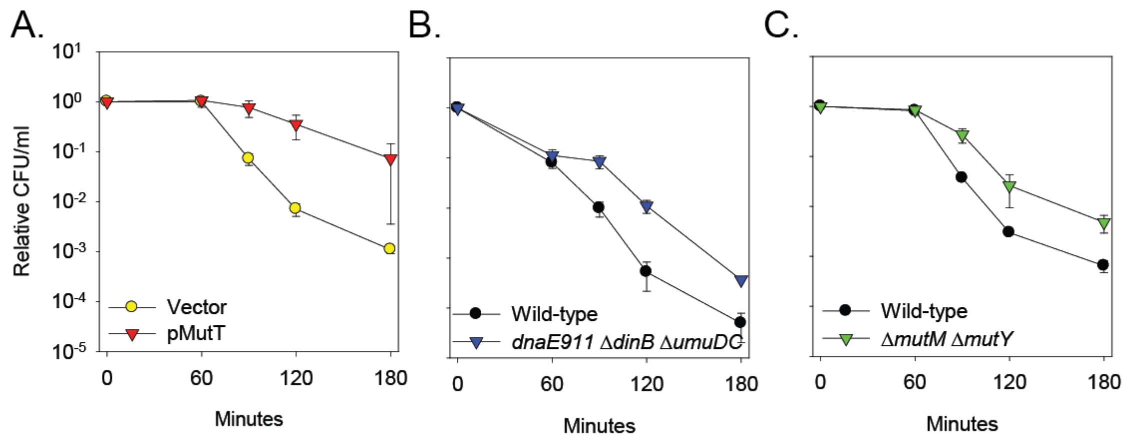

Fig. S4

Sensitivity of cells to killing by penicillin, the first antibiotic, is reduced by minimizing 8-oxo-dGTP incorporation and preventing GO-system glycosylase action. Measurement of relative colony forming units (CFUs) suggests that minimizing the incorporation of 8-oxo-dGTP by (A) over-production of MutT (pMutT; red) or (B) mutating three polymerases (*dnaE911*Δ *dinB* Δ*umuDC*; blue) reduces the penicillin sensitivity when compared to the vector control (yellow) or wild-type cells (black). Furthermore, deletion of the two (C) GO-system glycosylases (*mutM mutY*; green) reduces penicillin sensitivity compared to wild-type (black).

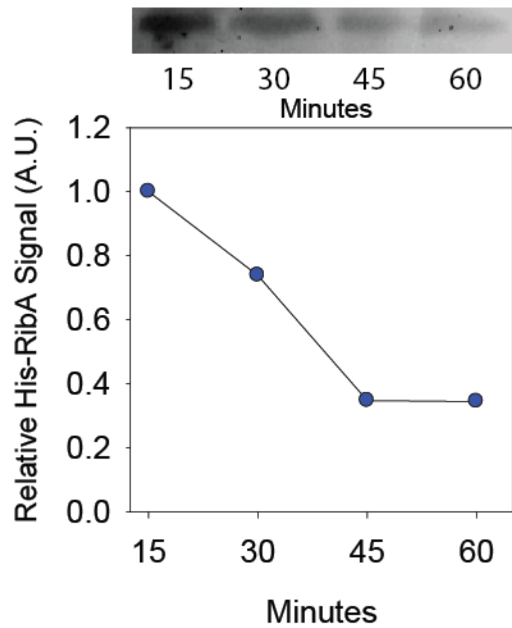

Fig. S5

RibA protein levels substantially decrease after protein synthesis is blocked. RibA-His levels from 15 to 60 minutes after kanamycin addition was detected by Western Blot and demonstrates the instability of the RibA protein (top). A plot of the relative His-RibA signal (arbitrary units; A.U.) versus time (bottom) was generated by quanitating the band intensity at each time point and comparing it to the 15-minute band signal.

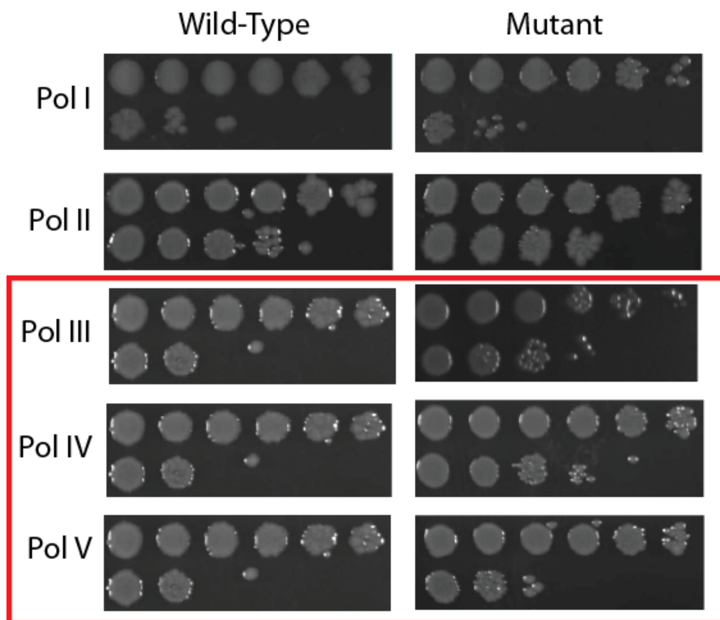

Fig. S6

Analysis of the effects of mutations affecting each of the 5 *E. coli* DNA polymerases suggest that Pol III, Pol IV, Pol V contribute to ampicillin-mediated cell death. Ten-fold serial dilutions of untreated (top rows) and treated (bottom rows) cultures of wild-type parental controls were plated [left column; Pol I: W3110 *thy*$^{-}$; Pol II: MG1655, and Pol III, Pol IV, and Pol V: AB1157] and compared to various polymerase mutants (right column: Pol I [*polA1*(Am)], Pol II [*polB*::frt Kan], Pol III [*dnaE911*; this allele reduces the mutation frequency of a Δ*mutT* mutant], Pol IV [*dinB*::frt cat], and Pol V [*umuDC595*::cat]). The red box around the Pol III, Pol IV, and Pol V results highlight the DNA polymerases that contribute to ampicillin-mediated cell death.

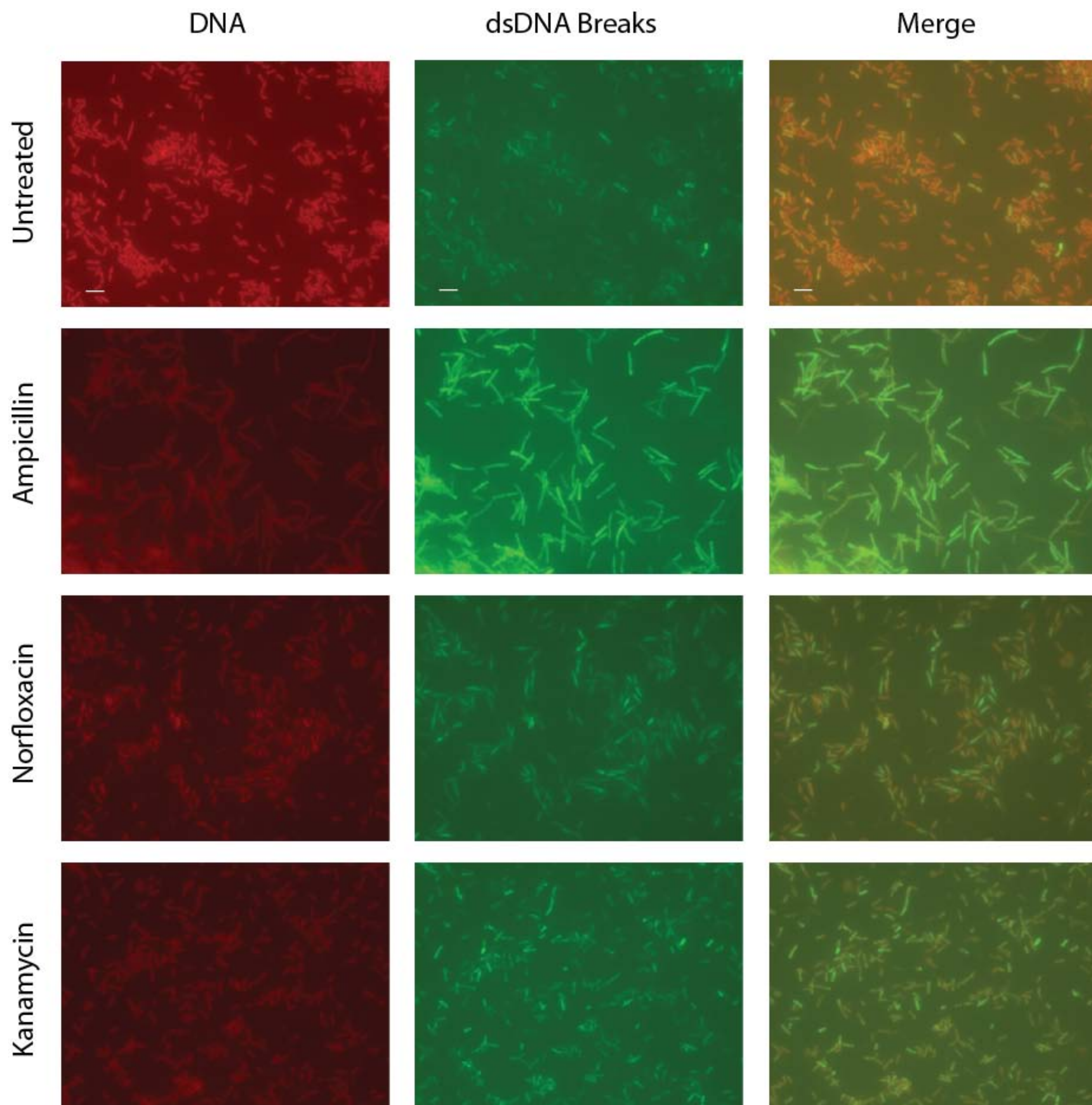

Fig. S7

The number of DSBs in wild-type *E. coli* cells increases after 30 minutes of treatment with bactericidal antibiotics. After 30 minutes of treatment, cells were stained for DSBs using the TUNEL assay and counterstained for DNA using a propidium iodine/RNase treatment. Representative images of the DNA (red) and dsDNA break (green) staining are shown with a merge of the two images. Bar is 5 μM.

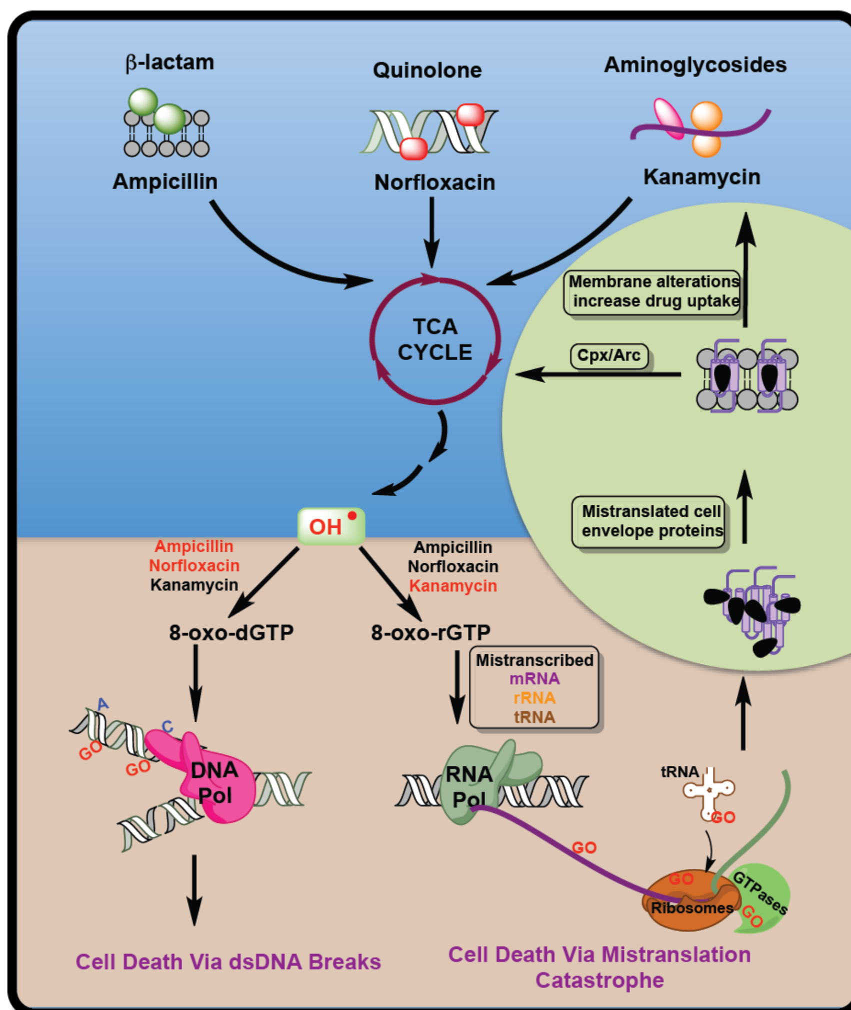

Fig. S8

The previously described common mechanism of cell death (shaded blue) induced by bactericidal antibiotics stimulates $OH^{\bullet}$ radical formation, which ultimately potentiates cell death (*1*). Although generalized oxidation of biomolecules probably contributes to cell death, the work described here suggests that oxidation of the guanine nucleotide pool to 8-oxo-guanine is a significant contributor to cytotoxicity (shaded brown). 8-oxo-dGTP formed via the oxidation of the deoxyguanine pool has a more prominent effect in mediating cell death for β-lactams and quinolones than for aminoglycosides. Once formed, closely spaced 8-oxo-dG lesions can be incorporated by several different DNA polymerases. The closely spaced 8-oxo-dG lesions could then potentially result in a lethal DSB via incomplete base excision repair. For aminoglycosides, cell death may additionally and predominantly be potentiated by oxidation of the rGTP pool and results in mistranslation due to 8-oxo-rG-containing mRNA, rRNA, and tRNA. Moreover, the function of essential GTPases could be altered due to a decreased capacity of these enzymes to hydrolyze 8-oxo-rGTP. Mistranslated cell envelope proteins can then result in further uptake of aminoglycosides and feed back into the $OH^{\bullet}$ producing pathway via Cpx and Arc as previously described (*35*) (shaded yellow), thereby leading to a catastrophic cycle of mistranslation driven by 8-oxo-rG.

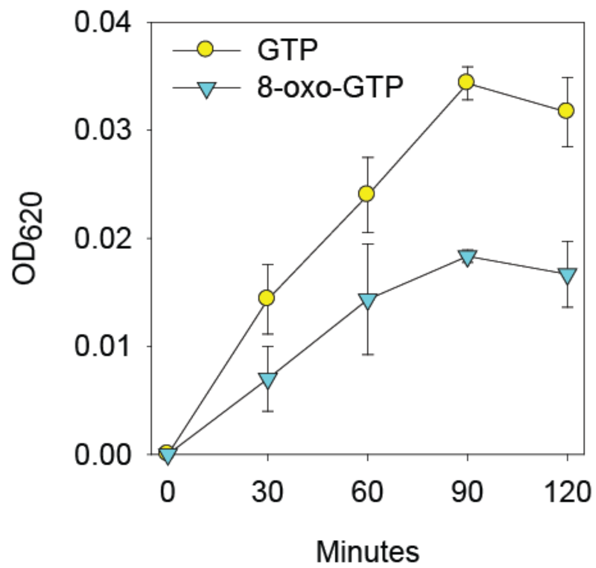

Fig. S9

Era GTPase has a reduced ability to hydrolyze 8-oxo-GTP compared to GTP. Era hydrolysis of 8-oxo-GTP and GTP was measured by adding the BioMol Green Reagent to a reaction containing Era protein and nucleotide at the indicated time. The BioMol Green Reagent was allowed to react with the free phosphate in solution resulting in a change in OD_{620}, thus allowing a measurement of Era hydrolysis (plotted as OD_{620} versus time).

Table S1.

Significantly Up-Regulated Genes During DinB Over-production

Category	Subcategory	Gene	Function	Delta Z-Score
SOS Response	*lexA+*	*dinB*	Pol IV	9.85
SOS Response	*lexA+*	*umuC*	Pol V catalytic subunit	4.71
SOS Response	*lexA+*	*umuD*	Pol V subunit	4.37
SOS Response	*lexA+*	*polB*	Pol II	4.14
SOS Response	*lexA+*	*sulA*	Inhibitor of cell division	2.84
SOS Response	*lexA+*	*yafO*	Toxin	2.75
SOS Response	*lexA+*	*recN*	Recombinational repair	2.67
SOS Response	*lexA+*	*yebG*	Unknown	2.65
SOS Response	*lexA+*	*yoaA*	Putative helicase	2.59
SOS Response	*lexA+*	*ymfJ*	Unknown	2.53
SOS Response	*lexA+*	*tisB*	Toxin	2.50
SOS Response	*lexA+*	*dinI*	Stabilizes RecA/ssDNA filaments	2.36
SOS Response	*lexA+*	*uvrB*	Nucleotide excision repair	2.33
SOS Response	*lexA+*	*dinD*	Unknown	2.20
SOS Response	*lexA+*	*dinF*	Unknown	2.19
SOS Response	*lexA+*	*recA*	SOS regulation and mutagenesis/homologous recombination	2.15
SOS Response	*lexA+*	*uvrD*	Nucleotide excision repair	2.11
SOS Response	*lexA-*	*recX*	Inhibitor of RecA	3.35
SOS Response	*lexA-*	*nrdB*	Ribonucleotide Reductase	3.22
SOS Response	*lexA-*	*nrdA*	Ribonucleotide Reductase	3.10
DNA Metabolism		*nrdG*	Ribonucleotide Reductase	3.42
DNA Metabolism		*nrdD*	Ribonucleotide Reductase	3.21
DNA Metabolism		*priA*	Replication Restart	2.24
DNA Metabolism		*nudK*	Nucleotide-sugar hydrolase	2.13
Prophage		*ymfT*	e14 prophage; predicated transcriptional regulator	2.86
Prophage		*ymfM*	e14 prophage; predicted protein	2.45
Prophage		*ymfL*	e14 prophage; predicted transcriptional regulator	2.53
Prophage		*ymfN*	e14 prophage; predicted transcriptional regulator	2.36
Prophage		*stfE*	e14 prophage; predicted side tail fiber protein fragment	2.17
Prophage		*ydaG*	Rac prophage; small protein	2.05
Biofilm		*ymgC*	protein involved in biofilm formation	3.23
Biofilm		*bdm*	biofilm-dependent modulation protein	2.47
Biofilm		*ymgA*	protein involved in biofilm formation	2.36
Miscellaneous Metabolism		*yfaE*	2Fe-2S cluster-containing protein	3.58
Miscellaneous Metabolism		*puuB*	γ-glutamylputrescine oxidase	3.24
Miscellaneous Metabolism		*puuC*	γ-glutamyl-γ-aminobutyraldehyde dehydrogenase	3.15
Miscellaneous Metabolism		*yqhD*	NADP-dependent dehydrogenase	2.44
Miscellaneous Metabolism		*gor*	glutathione reductase (NADPH)	2.43
Miscellaneous Metabolism		*yhiN*	predicted oxidoreductase with FAD/NAD(P)-binding domain	2.19
Miscellaneous Metabolism		*mdaB*	NADPH quinone reductase monomer	2.09
Miscellaneous Metabolism		*mnmE*	GTP-binding protein with a role in modification of tRNA	2.39
Miscellaneous Metabolism		*hepA*	RNA Polymerase (RNAP)-binding ATPase and RNAP recycling factor	2.15
Miscellaneous Metabolism		*rluA*	23S rRNA and tRNA pseudouridine synthase	2.13
Miscellaneous Metabolism		*ariR*	regulator of acid resistance, influenced by indole	2.71
Miscellaneous Metabolism		*mltB*	membrane-bound lytic murein transglycosylase B	2.45
Miscellaneous Metabolism		*glmU*	biosynthesis of UDP-*N*-acetyl-D-glucosamine	2.13
Miscellaneous Metabolism		*argE*	acetylornithine deacetylase	2.12
Miscellaneous Metabolism		*elbA*	inhibitor of σS proteolysis	2.06
Miscellaneous Metabolism		*fabB*	β-ketoacyl-ACP synthases	2.01
		ybhU	Unknown	3.02
		yidR	Unknown	2.29

Table S2.

Significantly Down-Regulated Genes During DinB Over-production

	Gene	Function	Delta Z-Score
Ion Metabolism	*feoC*	Fe-S dependent transcriptional regulator	-2.76
	clcA	Chloride ion channel	-2.64
	phoP	Transcriptional regulator activated in response to low extracellular levels of divalent cations	-2.64
	cvrA	Cation proton transporter	-2.47
	feoB	Ferrous ion transporter (fur regulated)	-2.33
	bfd	Bfr iron storage and release	-2.15
DNA Metabolism	*minE*	Restrict septation	-2.27
	hns	Histone-like nucleoid structuring protein	-2.26
	minC	Restrict septation	-2.15
	phr	DNA photolyase	-2.08
Miscallaneous Metabolism	*galR*	Galactose repressor	-2.92
	yegH	Predicted transporter	-2.68
	yoeA	CP4-44 prophage; predicted disrupted hemin or colicin receptor	-2.46
	yfiR	Putative swarming motility	-2.44
	rimJ	Ribosomal-protein-S5-alanine N-acetyltransferase	-2.27
	tyrA	Tyrosine biosynthesis	-2.26
	ycgF	Blue light-responsive regulator	-2.25
	yfjK	CP4-57 prophage	-2.22
	ycbB	Transpeptidase	-2.17
	dhaL	Dihyrdoxyacetone kinase	-2.12
	ygcI	BaeR regulated, which plays a role in novobiocin resistance	-2.11
	cls	Cardiolipin synthase	-2.10
	ychO	Predicted invasin	-2.09
	trpA	Tryptophan synthase	-2.04
	ydgA	Swarming	-2.03
	fruK	1-phosphofructokinase monomer	-2.02
	dacC	Penecillin binding protein required for cell morphology	-2.02
	idi	Biosynthesis of isoprenoids	-2.02
	yncA	Predicted acyltransferase	-2.01
	yghW	Unknown	-5.00
	yebS	Unknown	-2.33

Table S3.
List of Bacterial Strains

Strain	Background	Relevant Genotype	Source or Derivation
MG1655		F-	GCW Lab Stock
AB1157		F⁻ λ⁻ *his*G*4* *arg*E*3* *leu*B*6* *(gpt-proA)*Δ*62* *thr-1* *thi-1* *rps*L*31* *gal*K2 *lac*Y*1* *ara-14* *xyl-5* *mtl-1* *kdg*K*51* *sup*E*44* *tsx-33* *rfb*D*1* *mgl-51* *rac* *qsr'*	GCW Lab Stock
W3110 Thy⁻	W3110	F-, λ⁻, *e14-*, *thyA36*, *IN(rrnD-rrnE)1*, *deoC2*	(*45*)
P3478	W3110	F-, λ⁻, *e14-*, *thyA36*, *IN(rrnD-rrnE)1*, *pol*A*1*(Am), *deoC2*	(*45*)
NR9560	KA796	*mutL::*Tn*5* *dnaE*911 *zae*-502::Tn10	(28)
STL7255	MG1655	*recB*268::Tn10	(*46*)
JJF37	AB1157	Δ*dinB*::frt	Precise deletion (*47*) with marker removed
	AB1157	Δ*umuDC*595::cat	GCW Lab Stock
JJF217	MG1655	Δ*polB*::frt Kan	This work; Keio allele (*48*)
JJF242	AB1157	*dnaE*911 *zae*-502::Tn10	This work
DB200	MG1655	*dnaE*911 *zae*-502::Tn10 *dinB*::frt *umuDC*595::cat	This work
DB201	MG1655	Δ*recA*::frt	This work; Keio allele (*48*) with marker removed
DB202	MG1655	Δ*mutT*::frt	This work; Keio allele (*48*) with marker removed
DB203	MG1655	Δ*mutM*::frt Δ*mutY:*:frt	This work; Keio allele (*48*) with markers removed

References and Notes

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