MICROBIOLOGY

Drugging evolution of antibiotic resistance at a regulatory network hub

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Evolution of antibiotic resistance is a world health crisis, fueled by new mutations. Drugs to slow mutagenesis could, as cotherapies, prolong the shelf-life of antibiotics, yet evolution-slowing drugs and drug targets have been underexplored and ineffective. Here, we used a network-based strategy to identify drugs that block hubs of fluoroquinolone antibiotic-induced mutagenesis. We identify a U.S. Food and Drug Administration– and European Medicines Agency–approved drug, dequalinium chloride (DEQ), that inhibits activation of the *Escherichia coli* general stress response, which promotes ciprofloxacin-induced (stress-induced) mutagenic DNA break repair. We uncover the step in the pathway inhibited: activation of the upstream "stringent" starvation stress response, and find that DEQ slows evolution without favoring proliferation of DEQ-resistant mutants. Furthermore, we demonstrate stress-induced mutagenesis during mouse infections and its inhibition by DEQ. Our work provides a proof-of-concept strategy for drugs to slow evolution in bacteria and generally.

INTRODUCTION

Antibiotics have reduced mortality from bacterial infections (*1*), but, unfortunately, antibiotic resistance now threatens world health with an estimated 1.27 million deaths worldwide from antibiotic-resistant infections in 2019 (*2*). Evolution of resistance is outpacing introduction of new antibiotics (*3*, *4*). Drugs that could slow the rates of evolution, given with antibiotics, might prolong antibiotic effectiveness (*5*–*7*). Evolution-slowing drugs might, additionally, make antibiotics unnecessary if pathogen evolution could be slowed to rates lower than those of somatic evolution of our immune responses. This might allow immune clearance while avoiding the destructive effects of antibiotics on human microbiota, now appreciated to underpin many aspects of human health (*8*). Antibiotic resistance evolves via uptake of resistance genes from other bacteria and/or by mutations in native genes (*5*), with mutagenesis the main route in priority pathogens designated by the World Health Organization (*9*). Although potentially transformative (*6*, *10*–*15*), few mutagenesis-reducing drugs have been reported, and fewer drug targets identified (*7*, *16*–*18*). Moreover, those reported also reduce viability (*16*–*20*) and so favor proliferation of (select) mutants resistant to the evolution-slowing drug and the antibiotic [and see (*21*) for a similar anticancer drug].

We reasoned that ideal targets for evolution-slowing, or "anti-evolvability," drugs might include critical nonredundant hubs of functional protein networks that underpin antibiotic-induced mutagenesis [(*11*), reviewed in (*12*, *13*, *15*)]. See also (*10*, *22*–*24*) for mechanisms of antibiotic induction of mutagenesis for which protein networks are not known. Inhibition of hubs could prevent different parallel pathways to mutagenesis simultaneously (*11*). Here, we apply this strategy to mutagenesis induced by the widely used fluoroquinolone antibiotic (*22*) ciprofloxacin (cipro) (*10*, *25*).

Fluoroquinolones bind and inactivate bacterial type II topoisomerases mid-reaction, which kills cells via DNA double-strand breaks (DSBs) (*26*). Type II topoisomerases relieve replication- and transcription-generated positive DNA supercoils by cleaving opposite DNA strands, passing a duplex through, and then ligating the DSB ends (*27*). Both clinically and in the laboratory, fluoroquinolone resistance occurs mostly by de novo mutations that alter the topoisomerases, preventing drug binding, or up-regulate efflux pumps (*28*–*34*). Clinically isolated *Escherichia coli* that are cipro-resistant (mutants) occur at very high frequencies, ranging from 20% to 50% depending on the geographic area (*2*).

Cipro induces mutagenesis, which can generate resistance (*6*, *10*), including resistance to antibiotics not yet encountered (*35*). Cipro-induced mutagenesis occurs by mutagenic repair of DNA breaks, activated by the general stress (σ^S) response (*35*) (outlined in Fig. 1A) [reviewed (*15*)]. The mutagenesis functional protein network is described (*11*). At 20 million prescriptions per year in the United States (*25*), cipro is the second most prescribed antibiotic (after β-lactams) (*36*), and so is relevant clinically, in addition to its utility as a model. For example, a type II topoisomerase inhibitor, cipro inhibits bacterial topoisomerases similarly to the anticancer drug etoposide mechanism of action on human topoisomerases (*37*).

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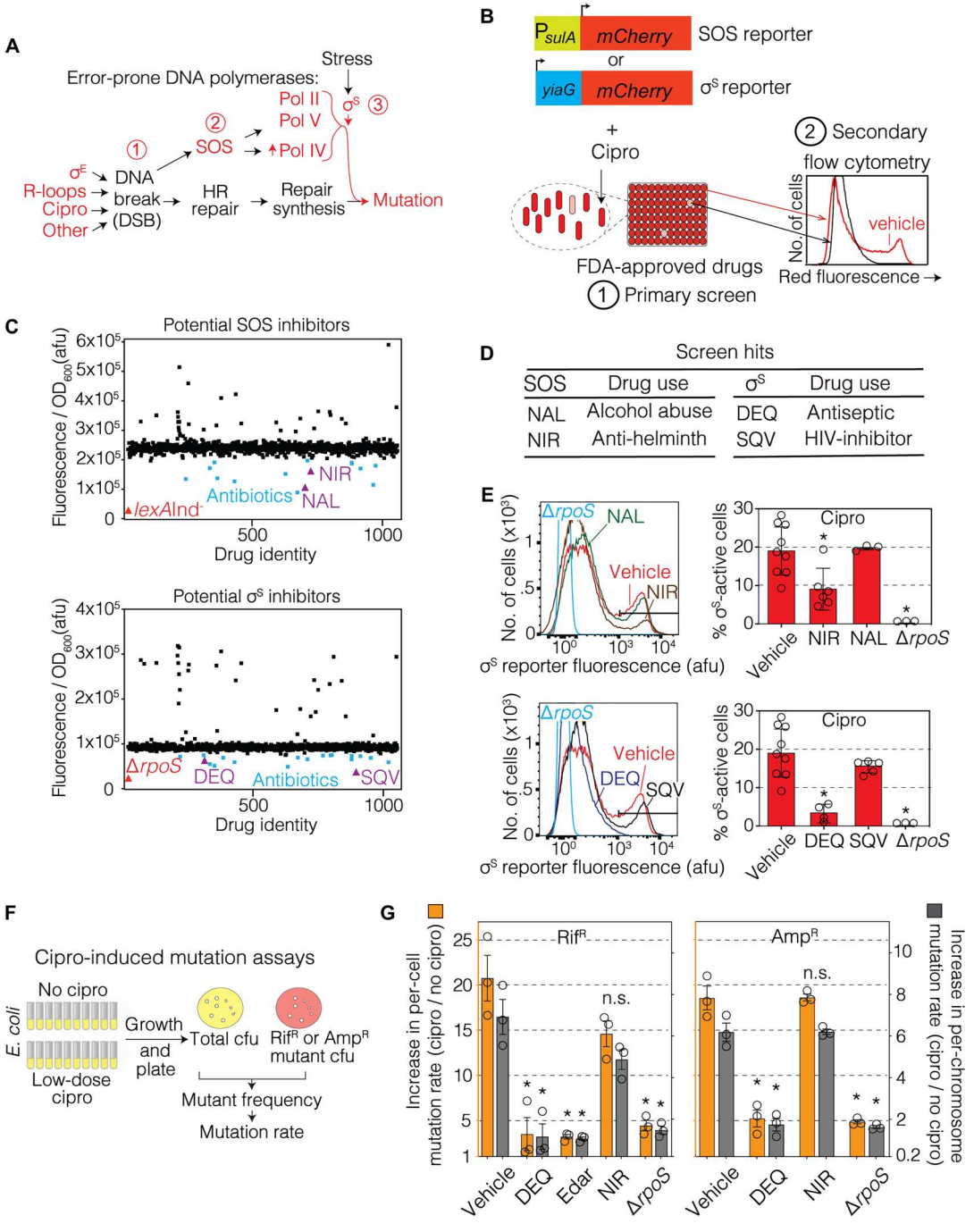

Fig. 1. DEQ inhibits cipro induction of the general stress response and mutagenesis. (**A**) Diagram of stress-induced mutagenic break repair (MBR) mechanism (*11*, *13*, *35*). Steps (1), (2), and (3) are necessary but not sufficient for MBR. (**B**) Design of screens for inhibitors of cipro-induced SOS or σ^S response activity, modified from (*75*). (1) Primary screen: fluorescence plate reader for drugs that reduce fluorescence from SOS or σ^S activity reporters. (2) Secondary screen: more accurate flow cytometry. (**C**) Data from plate reader screens in MAC cipro. Blue squares, antibiotics were not tested further; purple triangles, potential stress response inhibitors; red triangles, positive control SOS–uninducible *lexA*Ind$^-$ or σ^S response–uninducible Δ*rpoS* mutant cells. Strains: SMR24100, SMR24156, SMR24268, SMR24312. (**D**) Identities and approved uses of primary screen hits. SOS: Naltrexone (NAL), anti-addiction; niridazole (NIR), antischistosomal (antihelminth, anti-parasitic worms). σ^S: dequalinium chloride (DEQ) bacteriostatic at high doses unlike those here (*54*) (text S1); saquinavir (SQV) HIV protease inhibitor. (**E**) DEQ and NIR reduce cipro-induced σ^S-active cell subpopulation. Left, flow cytometry histograms, *yiaG-yfp* σ^S reporter. Black bar, gate for σ^S-active cells (Materials and Methods). Right, means ± SD, three independent experiments. Strains: SMR24096, SMR24134. (**F**) Fluctuation test assays for mutation rates of base substitutions (RifR) and any null mutation (AmpR) (*35*). (**G**) Cipro induction of mutagenesis is inhibited by DEQ. Fold induction of mutation rate: MAC cipro mutations per cell per generation (orange bars) or mutations per chromosome per generation (gray bars)/mutation rate without cipro. MAC cipro-treated cells contain ~4.5 chromosomes/cell, compared with 1.5 chromosomes/cell in no-drug controls (*35*). Means ± SEM, three experiments. Positive control edaravone (Edar): an antioxidant that inhibits mutagenesis (*35*). Baseline mutation rates (WT, vehicle no cipro) were $4.8 \pm 0.6 \times 10^{-9}$ RifR and $4.5 \pm 0.3 \times 10^{-9}$ AmpR mutations per cell per generation. *$P < 0.05$ (E), *$P < 0.001$ (G), one-way ANOVA with Tukey's post hoc test; n.s., not significant. afu, arbitrary fluorescence units. Strains: MG1655, SMR20479, SMR5223, SMR11641.

As a test case, we identify a drug, dequalinium chloride (DEQ), that inhibits the σ^S response, the central mutagenesis activator (Fig. 1A), and cipro-induced mutagenesis. A cipro-induced starvation (stringent) stress response (*38*) activates cipro induction of σ^S and mutagenesis (*39*) and is the drug's point of inhibition. At the low concentrations used here, 500 to 1000 times lower than the maximum tolerated oral dose in rat and mouse, respectively (*40*), DEQ does not increase cipro killing activity (text S1), which would favor proliferation of resistant mutants, and so is an apparent "stealth" (*15*) evolution-slowing drug. Furthermore, in mouse thigh infections, we demonstrate the occurrence of stress-induced mutagenesis similar to cipro-induced mutagenic break repair (MBR) in

culture. We show its reduction by this and another drug that inhibits its MBR, thus providing a proof-of-concept for drugs to slow evolution of drug resistance and escape from immune responses, found as network hub inhibitors.

RESULTS

Screen for stress response inhibitors finds anti-evolvability drug

The SOS DNA damage and σ^S general stress responses (*13*, *15*, *41*–*43*) are nonredundant hubs in an MBR network of more than 100 proteins (*11*, *44*, *45*). Most of these promote mutagenesis by sensing stress and transducing the signals to the stress response activators that up-regulate mutagenesis (Fig. 1A) (*11*). Cipro, a type II topoisomerase inhibitor, induces DSBs and SOS, which up-regulates error-prone DNA polymerases and proteins used in DSB repair. However, DSB repair remains nonmutagenic unless another stressor activates σ^S (*46*, *47*), e.g., starvation (*11*) or antibiotic exposure (*23*, *35*, *38*). σ^S is not needed for DSB repair (*46*–*48*) but allows error-prone DNA polymerase errors to occur in DSB repair (*48*) and to persist (*49*) causing mutations (Fig. 1A).

We screened a small-molecule library of 1120 approved-for-human-use drugs (Prestwick Chemical) for inhibition of the SOS or σ^S response. The drugs represent a large spectrum of chemical and pharmacological diversity (*50*). Cells with either an SOS or σ^S response fluorescence reporter gene (*11*, *51*, *52*) (Fig. 1, B to E) were grown in "sub-inhibitory" cipro at the minimum antibiotic concentration ("MAC," 10% viability; Materials and Methods), at which cipro-induced MBR is maximal (*35*). The primary screen for stress response inhibition used a fluorescence plate reader (Fig. 1, B to D; validated in fig. S1), which is high throughput but low resolution. A more accurate, single-cell secondary screen used flow cytometry (Fig. 1, B and E, and text S2), which examines single cells, and affords much higher resolution, and is low throughput.

The primary screen identified two potential SOS inhibitors and two potential σ^S inhibitors among 1120 compounds. Naltrexone (NAL) and niridazole (NIR) reduced apparent SOS response fluorescence, and saquinavir (SQV) and DEQ apparent σ^S response fluorescence (Fig. 1, C and D). NAL and NIR also inhibited growth of cipro-sensitive cells in cipro, an undesirable side effect (which selects resistance, see Introduction), whereas SQV and DEQ did not inhibit growth either in MAC cipro (fig. S2) or without cipro (fig. S2B). These experiments (fig. S2, A to C) mimic the experimental conditions of the mutagenesis experiments described in the following paragraph and used previously (*35*), in which MAC cipro is used here with or without DEQ, described in the fig. S2A legend.

In higher-resolution flow cytometry, none inhibited SOS (dose responses, fig. S3), whereas DEQ inhibited σ^S response activity (Fig. 1E, fig. S3, and text S2) and σ^S protein levels (fig. S4) robustly. Treatment with DEQ also caused cells with a σ^S protein fusion to β-galactosidase to display reduced β-galactosidase activity, which was not observed in cells with β-galactosidase produced from its native *lacZ* locus (fig. S4). NIR also partially reduced σ^S activation (Fig. 1E and fig. S3) and did not reduce cipro induction of reactive oxygen species (ROS) (fig. S5), which induce σ^S activity (*35*) (discussed in fig. S5).

We examined cipro-induced mutagenesis, as per (*35*), with assays for rifampicin or ampicillin cross-resistant mutants (RifR or AmpR) (Fig. 1F). These carry specific base substitutions in the *rpoB* gene or any null mutation in *ampD*. DEQ reduced cipro induction of mutation rates by 7.6-fold (±2.5) and 2.7-fold (±0.5, means ± SEM) for RifR and AmpR, respectively (Fig. 1G). NIR reduced neither significantly (Fig. 1G). Induction of mutation rates by cipro is shown as the rate with cipro/rate without cipro (Fig. 1G) and is estimated per cell per generation, and per chromosome per generation, because chromosome copies are increased in cipro-treated cells (*35*). We find that mutagenesis, not relative growth rate of RifR or AmpR mutant cells, is reduced by DEQ (fig. S2C). The data show that DEQ reduces mutagenesis, a validation of the strategy for identification of evolution-slowing drugs. DEQ (structure, fig. S2D) is used clinically at >100 times higher concentration than used here, with the antimicrobial clindamycin as a topical bacteriostatic agent to treat vaginal infection (*53*, *54*). Text S1 reviews the high bacteriostatic and low noninhibitory DEQ concentrations used here, which do not reduce cell viability (fig. S2, A and B). DEQ is well tolerated orally at 1000 mg/kg in rat and 2000 mg/kg in mouse (*40*), a concentration 1000 times higher than we used in mouse here, described below, and 100 times more than in culture (Figs. 1 to 4).

DEQ inhibits induction of small RNAs and the stringent response

We determined the step at which DEQ disrupts the pathway, previously elaborated (*35*, *39*), of cipro induction of the σ^S response and mutagenesis. As illustrated in Fig. 2A, cipro induces DSBs and the SOS response in all cells (quantified as foci and fluorescence, respectively) (*35*). The SOS response then induces ROS in a ~20% cell subpopulation (Fig. 2B) (*35*). The ROS are required for transcription of two small RNAs (sRNAs), DsrA and ArcZ (*35*), which promote translation of *rpoS* (σ^S) mRNA, activating the σ^S response. We found that DEQ prevented cipro induction of the *dsrA* and *arcZ* promoters (Fig. 2C and fig. S6) and did not reduce ROS-high cells (Fig. 2B). The ROS, σ^S response, and mutagenesis occur in a ~20% "gambler" cell subpopulation (Fig. 2, A and B) (*35*). Figure 2D illustrates the point in the pathway inhibited by DEQ.

Recently identified in the cipro-induced MBR pathway, the "stringent" starvation stress response is also activated in a roughly 20% cell subpopulation (*39*). The stringent-activated subpopulation cells are derived from the ROS-containing subpopulation cells, they lie downstream of ROS production, and are required for ROS induction of transcription of the sRNAs (*39*) (illustrated in Fig. 3A). The stringent response "on" subpopulation cells become (are the same cells as) the σ^S response–induced gambler cell subpopulation (*39*), which, when sorted by fluorescence-activated cell sorting (FACS), produces more than 90% of all cipro-induced mutants (*35*) (illustrated in Fig. 3A). Activation of the stringent response requires the nucleotide "alarmone" guanosine tetra- (or penta)-phosphate, (p)ppGpp, synthesized by the RelA and SpoT synthases (*55*) (positive controls; Fig. 3, B and C). We found that DEQ reduced stringent response activity assessed with the fluorescence reporter P_{rmf}*mCherry* (*39*) and flow cytometry (Fig. 3B), and also with the stringent response reporter P_{iraP}*lacZ*, in bulk cultures (Fig. 3C) (*56*). We conclude that DEQ interrupts the pathway to cipro-induced MBR after ROS (Fig. 2B) by reducing induction of the stringent response (Fig. 3, B and C), illustrated in Fig. 3A, which prevents downstream sRNA transcription (Fig. 2C) (*39*), σ^S induction (Fig. 1E) and mutagenesis (Fig. 1G). We reported previously

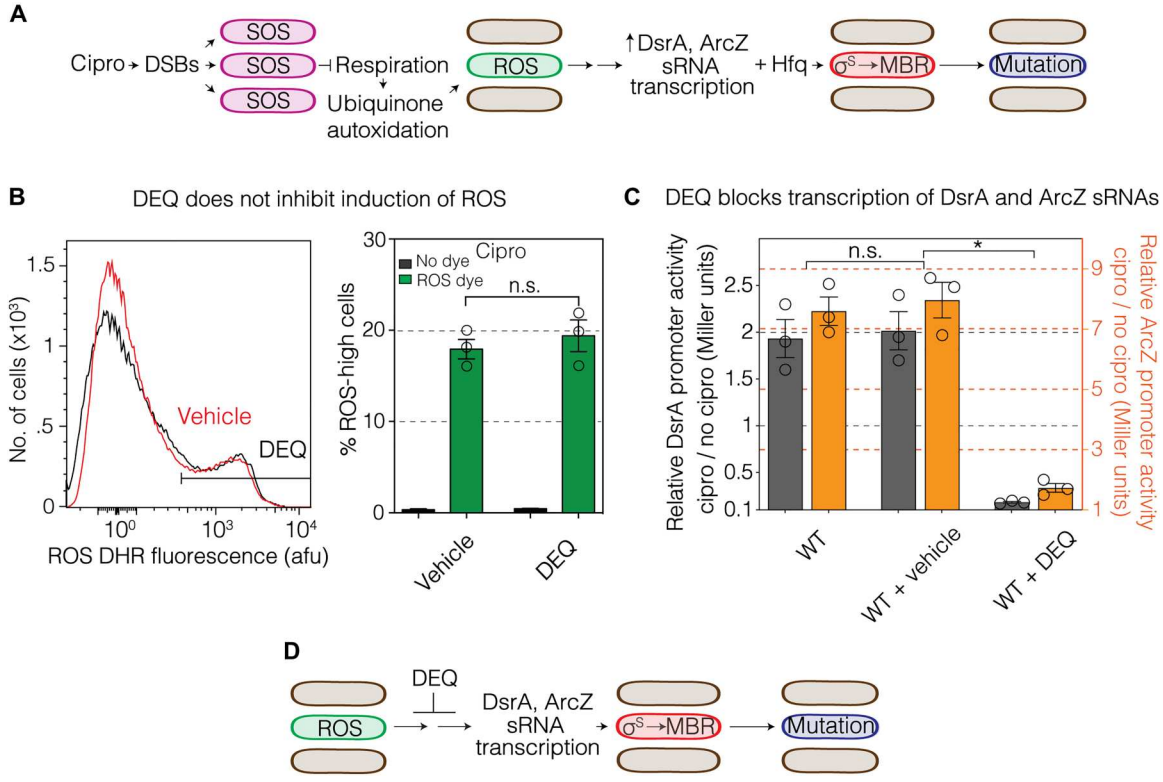

Fig. 2. DEQ interrupts induction of σ^S-activating small RNAs. (**A**) Summary diagram of observations of (*35*) that cipro induces DSBs and the SOS response in all cells; the SOS response then promotes ROS in a ~20% cell subpopulation; the ROS induce transcription of small RNAs (sRNAs) DsrA and ArcZ, which allow translation of *rpoS* mRNA into σ^S protein, thereby creating the σ^S-active "gambler" cell subpopulation that produces nearly all cipro-induced mutants via MBR (*35*). Ovals, *E. coli* cells. (**B**) Cipro-induced ROS-high cells are not reduced by DEQ. Flow cytometry of 16-hour log-phase cells grown in MAC cipro. DHR, dihydrorhodamine ROS dye. (B and C) Means ± SEM, three experiments. Two-tailed Student's *t* test. Strain: MG1655. (**C**) DEQ reduces cipro induction of *dsrA* and *arcZ* promoter activity. β-Galactosidase activity, P_{dsrA}-*lacZ*, and P_{arcZ}-*lacZ* reporters in log-phase growth in MAC cipro, ± DEQ. *$P < 0.001$, one-way ANOVA with Tukey's post hoc test. Strains: CH2046, PM1450. (**D**) Summary: DEQ inhibits DsrA/ArcZ sRNA synthesis and σ^S-active gambler cell formation and mutagenesis, but does not reduce ROS.

that the antioxidant drug edaravone inhibits cipro-induced mutagenesis by reducing ROS (*35*), a different step from that of DEQ shown here (Fig. 3A).

DEQ did not alter cell killing by cipro at a high therapeutic dose of 1.5 μg/ml (Fig. 3D), a level comparable with that in patients under cipro therapy (*57*) (text S1), and modeled here in mouse, discussed in the following section. We conclude that DEQ did not alter cipro antibiotic activity (Fig. 3D and fig. S2, A and C) or reduce cell growth rate with or without cipro (Fig. 3D and fig. S2, A and B). This implies that, in general, activation of the stringent or σ^S response might be points in the cipro-induced MBR pathway that can be inhibited without imposing strong selection for mutants resistant to the evolution-slowing drug (see Introduction and Discussion).

Stress-induced mutation and its drug inhibition occur in mouse infections

A key premise of dissection of the molecular mechanisms of antibiotic-induced mutagenesis is that, if found, stealth anti-evolvability drugs might slow evolution of resistance in living animals undergoing infection and antibiotic therapy. This potential strategy is supported by our demonstration here, first, that in an established and well-used preclinical mouse thigh infection model (*10*, *20*, *58*, *59*) (Fig. 4A), mutagenesis occurs in the infecting bacteria via a mechanism dependent on proteins of stress-induced MBR (Fig. 4B). In the mouse model, 10^6 CFU (colony-forming units) of bacteria are delivered into thigh wounds in neutropenic mice, followed in 2 hours by 1 mg/kg cipro to prevent death of the infected mice, and induce mutagenesis during infection. In our experiments, the candidate anti-evolvability drugs or vehicle were given with cipro. DEQ was given at a nontoxic 2 mg/kg (1000 times less than mice tolerate orally; text S1; and 100 times lower than its bacteriostatic dose). Cipro-treated bacteria were recovered after 2 days of infection and assayed for total CFU and RifR cross-resistant mutants (Fig. 4A); mice not given cipro died earlier (Materials and Methods).

We found that mutagenesis occurred in animals and was reduced to 6.3 ± 2.3 times lower than the control strain in isogenic bacteria defective for central stress response regulators of MBR: the SOS response (*lexA*Ind$^-$), σ^S response (Δ*rpoS*), and the Hfq RNA chaperone needed for σ^S induction by cipro (Fig. 4B) (*35*). The data are means ± SEM of three mice per strain, each in three independent experiments, averaged. The promotion of mutagenesis by stress response regulators demonstrates stress-induced mutagenesis [mutagenesis promoted by a stress response (*13*, *15*)] in bacteria during infection. Moreover, a mechanism similar to cipro-induced MBR is implicated by the dual requirements for SOS and the σ^S response (*15*, *35*). The mutant frequencies differ little

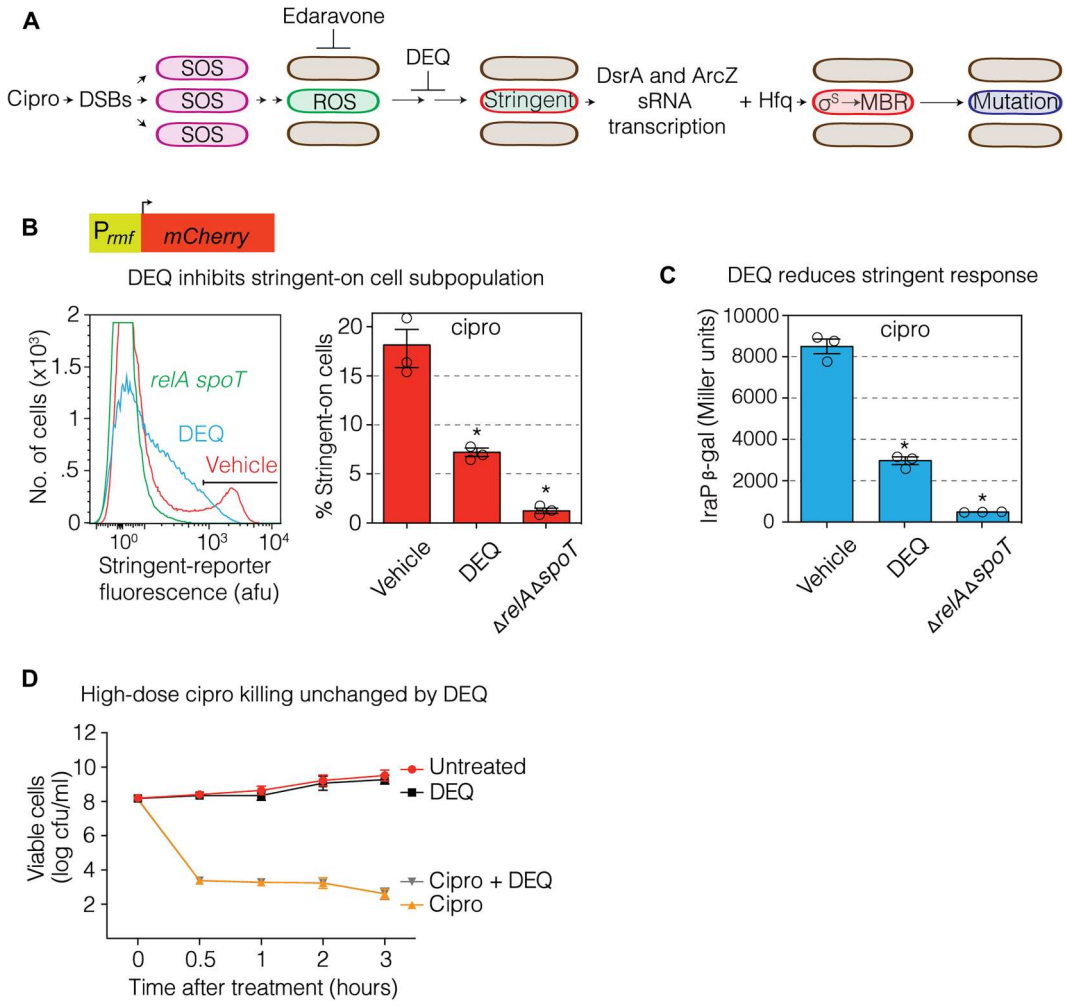

Fig. 3. DEQ inhibits cipro-induced stringent response and not antibiotic activity. (**A**) Summary: Edaravone and DEQ inhibit different steps in the newly expanded (*39*) MBR pathway (*35*). Antioxidant drug edaravone reduces the ROS-high cell subpopulation and MBR (*35*), whereas DEQ reduces cipro induction of the stringent response (*39*) (B) and (C). (**B**) DEQ reduces activation of the stringent response, transcriptional reporter P_{rmf}*mCherry* (*39*). The nucleotide (p)ppGpp is a stringent response activator that binds RNA polymerase (*60*–*62*). *relA spoT* mutants lack both *E. coli* (p)ppGpp synthases, and so are (p)ppGpp-deficient (*60*–*62*). Log-phase cells in MAC cipro (16 hours). Left, representative flow cytometry histograms; right, means ± SEM, three experiments. **P* < 0.001, one-way ANOVA with Tukey's post hoc test. Strains: SMR24273, SMR27009. (**C**) DEQ inhibits cipro-induced stringent-responsive *iraP* promoter activity. Log-phase cells in MAC cipro (16 hours). β-Galactosidase activity, P_{iraP}*lacZ* reporter. Bars as for (B). Strains: CH1623, CH6485. (**D**) DEQ does not increase high-dose cipro antibiotic killing activity. Log-phase cells grown with or without cipro (1.5 μg/ml). Means ± SD, four independent experiments. Strain: MG1655.

between those in mouse and those in culture (text S3), and the genetic dependencies of mutagenesis are the same (Fig. 4B) (*35*). To our knowledge, this is the first demonstration of σ^S response–dependent (stress-induced) mutagenesis in an animal infection model.

Strikingly, we found that both DEQ and edaravone reduced frequencies of RifR mutants robustly in mouse, causing reductions similar to those in the corresponding MBR-deficient mutants (Fig. 4B). These data show that small-molecule inhibitors (*35*) can slow bacterial evolution of resistance during infection, a demonstration of use of evolution-slowing drugs in animal infection, and example of the strategy generally.

DISCUSSION

Our data identify a drug, DEQ (fig. S2D), that targets the σ^S response, a mutagenesis network hub (*11*), and inhibits cipro antibiotic–induced mutagenesis (Fig. 1 and figs. S2 and S3), and identify the step at which it acts (Figs. 2 and 3, A to C) in a defined pathway (*35*, *39*) of cipro-induced mutagenic DNA break repair. DEQ blocks induction of the stringent starvation stress response (Fig. 3) after formation of its inducer, ROS (*39*) (Fig. 2B), and before stringent induction (Fig. 3, A to C) (*39*) of the two sRNAs (Fig. 2C) that activate σ^S in cipro-induced MBR, illustrated in Fig. 3A. Our data also provide evidence of stress-induced mutagenesis—mutagenesis upregulated by one or more stress response(s)—occurring in infection of an animal. DEQ reduced mutagenesis during mouse infections (Fig. 4B) and in culture (Fig. 1G). Moreover, when DEQ was used at 1000 times lower concentration in mouse than it is tolerated orally

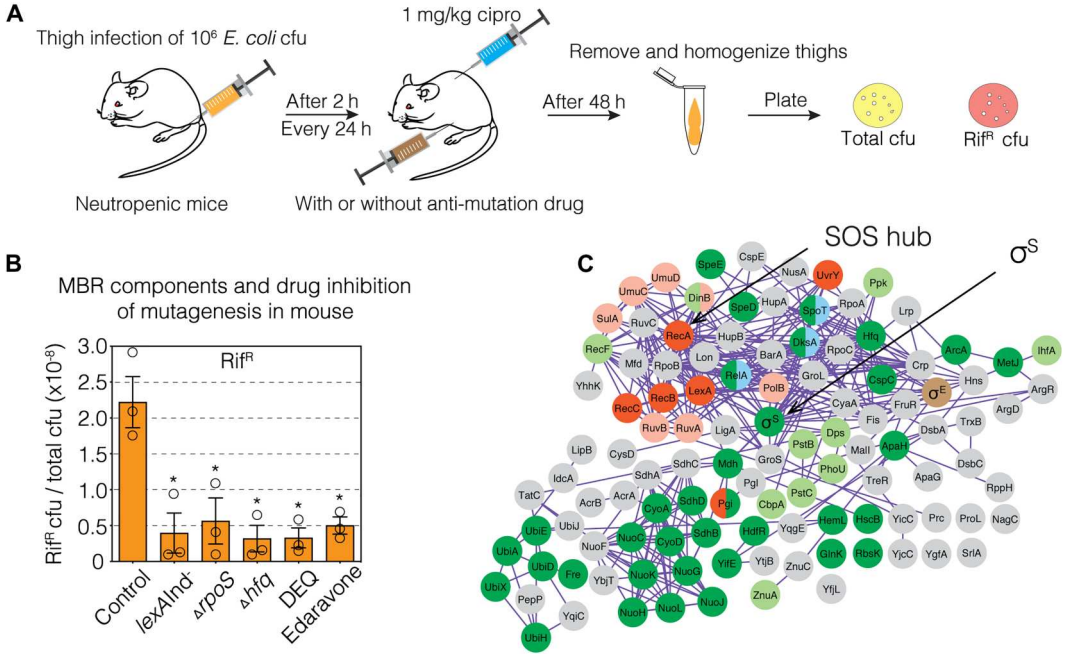

Fig. 4. MBR genes in mutagenesis in mouse infection and inhibition by DEQ and edaravone. (**A**) Workflow of bacterial mouse thigh infection model of (*59*). (**B**) Mutagenesis in mouse infection with cipro requires proteins/functions of cipro-induced MBR: an inducible SOS response (*lexA*Ind⁻ SOS-off mutant); σ^S (encoded by *rpoS*) and the Hfq RNA chaperone (*35*). Mutants reduced by 6.3 ± 2.3 times relative to the isogenic control strain. Means ± SEM of the three mutants, three biological replicates each. DEQ and edaravone inhibit mutagenesis to RifR antibiotic cross resistance during mouse infection by 6.4 ± 1.3 times and 3.7 ± 0.4 times, respectively. Means ± SEM, three experiments. Different from control, **P* < 0.01. One-way ANOVA with Tukey's post hoc test. ATCC 25922–derived *E. coli* cells extracted after 48-hour infection in mouse. Strains: RTC0021, SMR26989, SMR26991, SMR26993. (**C**) STRING interaction diagram (*76*) of known MBR network genes/proteins highlighting the SOS and σ^S responses, including the network of genes from (*11*), additional required components (*39*, *44*, *45*), and stringent response activators RelA, SpoT, and DksA (light blue), which act upstream of σ^S activation in cipro-induced MBR (*39*). Al Mamun *et al.* (*11*) estimated that their screen missed about half of the network. Green, shown previously (*11*, *39*) to act upstream of the σ^S response sensing stress and transducing signals that activate σ^S; light green, known MBR proteins up-regulated by σ^S; red, upstream regulators of the SOS response; and light red, MBR components up-regulated by SOS. The σ^E-activating components of the network (*11*) are not highlighted here; the σ^E activator hub is shown in brown. Two negative (upstream) regulators are noted: LexA repressor of the SOS response and ArcA, loss of which up-regulated σ^S (*11*).

(*40*) (text S4), and >100 times lower than its previous use topically (text S1), DEQ did not reduce bacterial cell viability or growth rate with or without cipro (Fig. 3D and fig. S2, A to C). At this low dose, DEQ is expected, therefore, not to favor proliferation of mutants resistant to DEQ or cipro: a "stealth" (*15*) evolution-slowing drug.

Antibacterial inhibitors of the stringent response have been identified previously and, unlike DEQ, were analogs of the stringent response activator (p)ppGpp, which binds RNA polymerase (*60*–*62*). Unlike DEQ, those drugs reduced viability, and so favor proliferation of resistant mutants. The (p)ppGpp analogs were developed as antibacterials, and their possible effects on mutagenesis were not examined. DEQ is not a (p)ppGpp analog (fig. S2D), and unlike the analogs, it inhibits the stringent transcriptional program (Figs. 2, B to D, and 3, B and C) with no measurable reduction of viability when used without cipro (Fig. 3D and fig. S2B), or in the presence of a therapeutic high cipro dose (Fig. 3D) or in MAC cipro (fig. S2A). MAC is defined as the drug concentration that produces 10% viable CFU (*63*). MAC is the most mutagenic cipro concentration (*35*) and will occur at the beginning and end of therapies, and when doses are missed (text S4). Possible mechanisms of the DEQ growth-neutral inhibition of stringent response transcriptional activity (Fig. 3D and fig. S2, A to C) are considered in text S5. We note that the DEQ dose that reduced mutagenesis in mouse here (Fig. 4B) is 2 μg/ml (or mg/kg), which is 1000 times lower than mice tolerate orally [2000 mg/kg (*40*); texts S1 and S4], and 100 times lower than the dose we used in culture, throughout, and so might be expected also not to impair bacterial viability in animals. Viability cannot be measured directly in mouse.

New antibiotics designed to bind two different target molecules were developed to resist resistance by requiring, presumably, two independent mutations for resistance, one in each of the two targets' gene (*64*). Cipro also binds two targets, topoisomerases II and IV (*37*), and one way cells become resistant is by up-regulation of efflux pumps (*5*), which export the drug. The frequencies of mutants resistant to the new drugs are not yet known. Whether or not they are unusually low, combining new or old antibiotics with evolution-slowing drugs might resist resistance robustly.

More uniquely, evolution-slowing drugs such as DEQ might make possible treatment of infections without antibiotics. Slowing pathogen evolution might allow immune response somatic evolution to outstrip the pathogen to allow clearance of infections without harm to the native human microbiome, e.g., (*15*). The microbiome underpins many aspects of human health (*8*). The possibility of efficacy without antibiotic cotherapy has not been tested for DEQ, or, to our knowledge, any mutagenesis-reducing drug, and would require a different mouse infection model than used here.

Whether DEQ (or edaravone) would be useful for slowing bacterial evolution clinically is unknown and requires future clinical

studies. However, both support the proposal that nonkilling (*35*) (Fig. 3D and fig. S2, A to C) stealth (*15*) evolution-slowing drugs can be identified by functional screens for reduced activity of the σ^S general stress response.

Figure 4C illustrates the MBR protein network (*11*, *39*, *44*, *45*) and its SOS and σ^S response activators, which are critical nonredundant hubs (*11*). Hubs coordinate multiple inputs that "fan into" a common node (*65*). Inputs shown to act upstream of σ^S activation in MBR (*35*, *39*) are shown as dark green circles in Fig. 4C. At hubs, a common "currency" of few information carriers is used to "fan out" into various outputs (*65*); those known for σ^S and relevant to MBR are shown as light green circles (Fig. 4C). Such "bow-tie" informational architectures are robust to perturbations without losing whole-system function and are flexible to evolution of new inputs and outputs (*65*), but their inherent weakness is their hubs or "knots," disruption of which can shut a system down. The hubs in stress-induced MBR arethe stress response activators and are potentially useful targets for evolution-slowing drugs (*11*) and their identification by function-based chemical genetic screens.

Our data also provide evidence of stress-induced mutagenesis—mutagenesis up-regulated by one or more stress response(s)—occurring during infection. Stress-induced MBR is one of the most well-characterized molecular mechanisms of spontaneous or drug-induced mutagenesis based on work in culture, with more than 100 proteins implicated in the MBR protein network (Fig. 4C) [(*11*), reviewed in (*13*, *15*)]. The apparent occurrence of stress-induced MBR in animal infections identifies a vast trove of potential targets for design of future anti-evolvability drugs against bacteria. Whether any will work clinically is not known. All caveats apply concerning the major difficulties of translating from animal models to clinical utility. However, given that the antibiotic kills by >5 to 6 logs, the fate of the much smaller remaining bacterial population may be clearance by the immune system or generation of new mutations to resistance (*66*, *67*). Those new mutations are expected to be fewer, making clearance more likely before rebound of a resistant (mutant) population. For mutation rates of ~10^{-6} per living gambler cell (*35*), knocked down ~10-fold by DEQ, this will matter most when fewer than ~10^7 gamblers (5×10^7 total bacteria) are present. Of course, evolution-slowing drugs would not affect infections of already resistant bacteria, which are not killed by cipro, not stressed, and do not require new mutations for proliferation.

In addition, there are multiple recent demonstrations of stress-induced mutagenesis mechanisms accelerating the development of human cancers (*68*–*70*). Those findings suggest that similar evolution-slowing drugs might be considered as future preventatives against cancers (*12*).

MATERIALS AND METHODS

Bacterial strains, media, and growth

E. coli strains used and their origins are given in table S1. Assays for rifampicin-resistant (RifR) and ampicillin-resistant (AmpR) mutants were performed in one of two "wild-type" (WT) *E. coli* strains and their isogenic derivatives. Bacteria were grown in Luria Bertani Herskowitz (LBH)-rich medium at 37°C with aeration, and additives, where indicated, at the following concentrations: cipro (1 to 8.5 ng/ml; table S2), ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), rifampicin (110 μg/ml), sodium citrate (20 mM), and 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). MAC is the concentration at which 10% of treated cells remain as viable CFU, compared with cultures with no antibiotic (*63*), and was determined for each strain experimentally, as described previously (*35*).

Drug screens

We used the Prestwick Chemical Library from Prestwick Chemical (Illkirch, France), which contains off-patent approved-for-human use drugs, a gift from A. Kuspa laboratory. The strain SMR24268 with the *yiaG-mCherry* σ^S response reporter (*11*, *35*) or SMR24100 with the Δ*att*λ::P_{sulA}*mCherry* SOS reporter (*52*), modified from (*51*), was used for the drug screens. Saturated overnight LBH cultures, started each from a single colony, were diluted 1:4 × 10^6 into 25 ml in a 250-ml flask in fresh LBH broth and incubated at 37°C with shaking for 3 to 3.5 hours. Seventeen microliters of each drug, 1 replicate, at 8 μg/ml (2× final concentration per Prestwick Chemical recommendations for screening concentration), or vehicle [2% dimethyl sulfoxide (DMSO), 2× final concentration], was pipetted into each well of a 384-well plate, followed by addition of 17 μl of the following mixture: cells (in LBH) carrying either the *yiaG-mCherry* σ^S response reporter or the Δ*att*λ::P_{sulA}*mCherry* SOS reporter, plus cipro (17 ng/ml, which is 2× final concentration) or vehicle. To reduce effects from evaporation, the outside rows and columns of each plate were filled with medium but were not used. Also, on each plate screened, *lexA*Ind$^-$ cells (SOS response uninducible) or Δ*rpoS* cells (no σ^S activity) were used as genetic positive controls. After 15 hours of growth, the SOS inhibitor screen was analyzed on a BioTek plate reader (Winooski, VT). The σ^S response inhibitor screen was analyzed after 36 hours of growth. For both screens, total mCherry fluorescence and OD_{600} (optical density at 600 nm) were measured. Z-factors were calculated for each assay as described (*71*). Hits for further analysis met the following criteria: (i) not a known antibiotic drug, and (ii) inhibiting cipro activation of either the SOS response or σ^S response. For secondary flow cytometry validation, new drugs were obtained from Sigma-Aldrich to rule out potential chemical contamination of the drug screening library. The molar concentrations of the hit compounds in the screen were as follows: NIR, 1.3 μM; DEQ, 30 μM.

Assays for cipro-induced mutagenesis

Assays for rifampicin-resistant (RifR) and ampicillin-resistant (AmpR) mutants were performed with cipro at MAC in the WT *E. coli* strains MG1655 and SMR5223, and their isogenic derivates, as previously (*35*). For fluctuation tests performed with the addition of drugs identified in our screen, the final concentrations were 30 μM for DEQ and 1.3 μM for NIR. Ten to 60 aliquots of log-phase cultures were diluted 1:3 and dispensed into 14-ml tubes with and without the identified drugs and with and without MAC cipro, and then grown at 37°C shaking for the mutagenesis assays for 24 hours (RifR) or 48 hours (AmpR) and assayed for mutant and total CFU, per (*35*). Mutation rates were estimated with the MSS-MLE algorithm using the FALCOR calculator (*72*).

Flow cytometry for SOS, σ^S, and stringent response activity

Flow cytometric assays for SOS, σ^S, and stringent response–regulated promoter activity were as described (*35*, *39*). Quantification of cells that have induced their SOS, stringent, or σ^S response, and how much they have, was achieved using engineered chromosomal

fluorescence reporter genes and flow cytometry, per (*51*, *52*) for SOS, per (*11*, *35*) for σ^S response activation, and per (*39*) for stringent response activation, using Δ*attλ*::P_{sulA}*mCherry* SOS reporter (*52*) modified from (*51*) for SOS, the *yiaG-yfp* σ^S response reporter (*11*), and the P_{rmf}*mCherry* stringent response reporter (*39*). Strains were grown under fluctuation test conditions as described in the previous section, with or without cipro, at indicated concentration(s), and were harvested in late log phase or stationary phase. For quantification, flow cytometry "gates" were calibrated, for SOS, using the negative control SOS-off *lexA*(Ind$^-$) and SOS response–proficient cells (*51*) as the dividing place between peaks of the distribution of SOS-proficient cells at which most cells diverge from the spontaneously SOS-induced fluorescent cell subpopulation, usually at between 0.5% and 1% of cells cultured in LBH broth. With this gate, ~10^{-4} of SOS-uninducible *lexA*Ind$^-$ cells cross the gate, scoring as "SOS-positive" (*51*). For the σ^S response, gates were set to the point at which fewer than 0.5% of cells with cipro but without the reporter gene were positive. At this gate, fewer than 10^{-3} of Δ*rpoS* cells, which are σ^S response deficient, cross the gate and would be scored as positive. For all, the percent of the population that scored as positive is reported. For the secondary confirmation of each drug, we used at least four doses relative to the molar concentration used in the initial hit screen (see fig. S3, A to D). For the stringent response, gates for stringent response–active cells were set to the point at which fewer than 0.5% of cells with cipro but without the reporter gene were positive.

Single-cell detection of intracellular ROS by flow cytometry

Cells were grown in the absence or presence of cipro at its MAC (8.5 ng/ml) to log phase as in the "Assays for cipro-induced mutagenesis" section. The ROS measurement protocol was adapted from Pribis *et al.* (*35*). Cells were grown in the presence of MAC cipro with or without the identified drug as in the "Assays for cipro-induced mutagenesis" section and then harvested serially from cultures for ROS detection using dihydrorhodamine (DHR; Life Technologies).

β-Galactosidase assays

Cells were grown as in the "Assays for cipro-induced mutagenesis" section to equivalent ODs and frozen at −20°C until assays were carried out. Determination of the β-galactosidase activity of the P_{dsrA}-*lacZ*, P_{arcZ}-*lacZ*, *rpoS-lacZ*, and P_{iraP}-*lacZ* fusion strains was accomplished using the standard assay described by J. H. Miller, as described previously (*35*, *73*, *74*).

Measurement of high-dose cipro antibiotic activity

Cells were grown to log phase OD_{600} ~ 0.5, and then cipro (1.5 μg/ml) was added with or without DEQ (30 μM) and returned to 37°C incubation shaking. Cells were harvested 0.5, 1, 2, and 3 hours later to determine CFU/ml. Cells were washed twice with phosphate-buffered saline (PBS) and then assayed for viable CFU on LB plates.

Neutropenic murine bacterial thigh infection model

Animal care and experimental procedures were approved by Baylor College of Medicine Institutional Animal Care and Use Committee in accordance with all guidelines set forth by the U.S. National Institutes of Health. The protocol for neutropenic bacterial thigh infection was modified from (*10*, *20*, *59*). Before cyclophosphamide treatment began, mice were administered buprenorphine at 1 mg/kg. Six- to 8-week-old female CD-1 mice (Charles River Laboratories; weight: 25 to 35 g) were rendered neutropenic by intraperitoneal injection of 150 mg/kg cyclophosphamide (Sigma-Aldrich) 4 days before infection and 100 mg/kg cyclophosphamide 24 hours before infection. LB cultures inoculated from fresh *E. coli* [American Type Culture Collection (ATCC) 25922] colonies were grown to the log phase (OD_{600} approximately 0.3) and diluted in LB broth. Thigh infections were performed by injecting 50 μl (approximately 10^6 CFU) of diluted cultures into the thigh muscle of isoflurane anesthetized mice. Starting 2 hours after infection (defined as time zero), mice were administered subcutaneous injections of 1 mg/kg of the antibiotic cipro, or vehicle, and intraperitoneal injections of 2 mg/kg DEQ,10 mg/kg edaravone, or vehicle every 24 hours for 2 days. Animals given vehicle with no cipro died from untreated infection. Cipro-treated mice were monitored daily for signs of distress. After 48 hours, cipro-treated mice were euthanized, and thighs of each were removed and homogenized in groups of three thighs combined, which were then resuspended in 1 ml of PBS buffer, from which serial dilutions were plated on LB agar containing kanamycin for total CFU, and undiluted homogenates plated on LB agar containing kanamycin and rifampicin for RifR CFU. Total bacterial colonies were enumerated following 24 hours of incubation, RifR colonies were enumerated following 48 hours of incubation, and the frequency of viable RifR mutants per viable CFU was calculated. We typically observed 1.1 (± 0.2) × 10^9 total CFU and 25 ± 4 RifR CFU per three thighs at 48 hours from cipro-treated mice given control bacteria. SOS-defective bacteria showed fewer total CFU at 48 hours. DEQ-treated mice had 1.5 (± 0.3) × 10^9 total CFU and 4 ± 0.3 RifR CFU per three thighs. Data are means ± SEM of three independent experiments of three mice each.

Statistics

For comparisons of two groups, a two-tailed Student's *t* test was used. For comparisons of three or more groups, analysis of variance (ANOVA) with Tukey post hoc test was used. Statistics were performed using GraphPad Prism.

Supplementary Materials

This PDF file includes:

Supplementary Texts S1 to S5
Figs. S1 to S6
Tables S1 and S2
References

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