Fast Evolution of SOS-Independent Multi-Drug Resistance in Bacteria

Le Zhang[1,†], Yunpeng Guan[1†],Yuen Yee Cheng[1], Nural N. Cokcetin[2], Amy L. Bottomley[2], Andrew Robinson[3], Elizabeth J. Harry[2], Antoine van Oijen[3], Qian Peter Su[1#], Dayong Jin[1#]

[1]Institute for Biomedical Materials and Devices (IBMD), University of Technology Sydney, Ultimo, Australia.

[2]Australian Institute for Microbiology & Infection (AIMI), University of Technology Sydney, Ultimo, Australia.

[3]School of Chemistry and Molecular Bioscience, University of Wollongong, Wollongong, Australia.

†These authors contributed equally to this work

[#]Corresponding author. Email: qian.su@uts.edu.au; dayong.jin@uts.edu.au

Abstract

The killing mechanism of many antibiotics involves the induction of DNA damage, either directly or indirectly, which triggers the SOS response. RecA, the master regulator of the SOS response, plays a crucial role in driving the evolution of resistance to fluoroquinolone antibiotics treated with a single dose of ciprofloxacin. However, the precise roles of RecA and SOS responses in the development of resistance under short-term β-lactam exposure remain unclear. In the present study, we observed a fast evolution of β-lactam resistance (20-fold increase in MIC in 8 hours) in *E. coli* after deleting RecA and exposing the bacteria to a single dose of ampicillin. Notably, once this type of resistance is established, it remains stable and can be passed on to subsequent generations. Contrary to previous findings, it is shown that this accelerated resistance development process is dependent on the hindrance of DNA repair, which is completely orthogonal to the SOS response. Additionally, we identified the rapid emergence of drug resistance associated mutations in the resistant bacterial genome, indicating the impairment of DNA repair. Through comprehensive transcriptome sequencing, we discovered that the expression of numerous antioxidative response genes is repressed in *recA* mutant resistant isolates, resulting in an excessive accumulation of ROS within the cells. This suggests that the induction of ROS drives the fast evolution of antibiotic resistance in RecA-deficient bacteria. Collectively, we show that the hindrance of DNA repair hampers cellular fitness, provides bacteria with genetic adaptability to survive in diverse stressful environments, and accelerates the evolution of antibiotic resistance.

Introduction

Addressing bacterial infections caused by emerging and drug-resistant pathogens represents a major global health priority. Bactericidal antibiotics can exert their effects on cells by directly or indirectly causing DNA damage or triggering the production of highly destructive hydroxyl radicals (1-3). This, in turn, initiates a protective mechanism known as the SOS response, which enables bacterial survival against the lethal impacts of antibiotics by activating intrinsic pathways for DNA repair (4-7). The activation of DNA repair processes relies on specific genes, such as *recA*, which encodes a recombinase involved in DNA repair, and *lexA*, a repressor of the SOS response that can be inactivated by RecA (8).

Studies have demonstrated that a single exposure to fluoroquinolones, a type of antibiotic that induces DNA breaks and triggers the SOS response, leads to the development of bacterial resistance in *Escherichia coli* (*E. coli*) through a RecA and SOS response-dependent mechanism (9). Given the crucial role of RecA in the SOS response, inhibiting RecA activity to deactivate the SOS response presents an appealing strategy for preventing the evolution of bacterial resistance to antibiotics (10). Similarly, exposure to fluoroquinolones induces the SOS response and mutagenesis in *Pseudomonas aeruginosa*, and the deletion of *recA* in this pathogen results in a significant reduction in resistance to fluoroquinolones (11).

Unlike fluoroquinolones, β-lactam antibiotics induce a RecA-dependent SOS response in *E. coli* through impaired cell wall synthesis, mediated by the DpiBA two-component signal system (12). The development of antibiotic resistance, triggered by exposure to β-lactams, has been extensively investigated using the cyclic adaptive laboratory evolution (ALE) method. Mutations that arise during cyclic ALE experiments are attributed to errors occurring during continued growth, necessitating multiple rounds of β-lactam exposure to drive the evolution of resistance in *E. coli* cells (13,14). However, the precise roles of RecA and SOS responses in the development of resistance under short-term β-lactam antibiotics exposure remain unclear.

Recently, there has been a growing interest in understanding the impact of the stress-induced accumulation of reactive oxygen species (ROS) on bacterial cells (15,16). While exploring methods to harness ROS-mediated killing has the potential to enhance the effectiveness of various antibiotics (17-19), the role of ROS in antimicrobial activity has become a topic of controversy following challenges to the initial observations (20,21). The generation of ROS has been found to contribute to the development of multidrug resistance, as prolonged exposure

to antibiotics in cyclic ALE experiments is known to generate ROS, leading to DNA damage and increased mutagenesis (22,23). Nevertheless, there is still limited knowledge regarding the consequences of ROS accumulation in bacteria when the activity of RecA or the SOS response is suppressed. Here, in the present work, we observed a fast evolution of multi-drug resistance in an *E. coli* strain with the *recA* mutation, following a single exposure to β-lactam antibiotics. This evolution can be attributed to three mechanisms: compromised DNA repair, transcriptional repression of genes associated with antioxidative processes, and the excessive accumulation of ROS.

Results

Single exposures to β-lactam antibiotics trigger a fast evolution of antibiotic resistance in the *recA* mutant strain

To investigate the impact of the SOS response on bacterial evolution towards β-lactam resistance, we generated a *recA* mutant strain (*ΔrecA*) from the *E. coli* MG1655 strain (LZ101). Initially, we conducted an ALE experiment using a slightly modified treatment protocol (24) on the wild type and *ΔrecA* strains. During a period of three weeks, the cells were subjected to cycles of ampicillin exposure for either 4 or 8 hours at a concentration of 50 µg/mL (10 times the MIC) each day (Fig. S1A) (25). As anticipated based on previous studies, the intermittent ampicillin treatments over the course of three weeks resulted in the evolution of antibiotic resistance in the wild type strain (Fig. S1B and C). However, we observed rapid development of ampicillin resistance in the *ΔrecA* strain after a single exposure for 8 hours to ampicillin (Fig. 1A-C). To ensure that the emergence of resistance we observed was not illusory due to technical issues during the *recA* knockout process, we employed another *ΔrecA* strain (JW2669-1) provided by the Coli Genetic Stock Centre (CGSC) with the same killing procedure. The results from both bacterial strains were consistent (Fig. S2A and B). To further investigate, we treated both the wild type and *ΔrecA* cells with other β-lactams, including penicillin G and carbenicillin, at concentrations equivalent to 10 times the MIC (1 mg/mL and 200 µg/mL, respectively) for 8 hours (26,27). Consistently, these treatments also led to a fast evolution of antibiotic resistance in the *ΔrecA* strain (Fig. S3A and B).

To assess the stability of this accelerated antibiotic resistance acquired by the *ΔrecA* strain, we conducted a study wherein the *ΔrecA* resistant isolates, originating from the initial 8-hour treatment with ampicillin, were continuously cultivated in a medium devoid of antibiotics for a period of seven days. Our findings revealed that once resistance was established, resistance

remained stable and was able to be passed on to subsequent generations even in the absence of ampicillin (Fig. 1D). Moreover, we performed a complementation experiment by introducing a plasmid containing *recA* under its native promoter into the *ΔrecA* strain prior to Step i in Fig. 1A, that is, before exposing the cells to ampicillin. Interestingly, this complemented strain displayed a comparable MIC to the isogenic wild type strain and maintained its sensitivity even after ampicillin treatment for up to 8 hours (Fig. 1E).

Notably, the evolution of antibiotics is influenced by mutation and selection, among other factors. To further investigate the primary drivers behind the rapid development of antibiotic resistance, we employed rifamycin, an unrelated antibiotic. Our findings indicated that following an 8-hour exposure to ampicillin, the MIC of rifampicin reached about 100 μg/mL for the *ΔrecA* strain, marking a 10-fold increase compared to the wild-type strain (Fig. 1F). Moreover, we measured and calculated the mutation frequency by employing rifampicin as the selective agent in both the wild type and *ΔrecA* strains after the 8-hour ampicillin exposure. This was done by dividing the number of colony-forming units (CFU) per milliliter on ampicillin plates by the number of CFU per milliliter on LB plates (28). Our results revealed a significantly higher mutation frequency in the *ΔrecA* strain in contrast to the wild type (Fig. 1G). These findings together demonstrate that a singular exposure to β-lactam antibiotics markedly boosts mutation frequency and thereby triggers a fast evolution of antibiotic resistance that is stable and heritable in the *ΔrecA* strain.

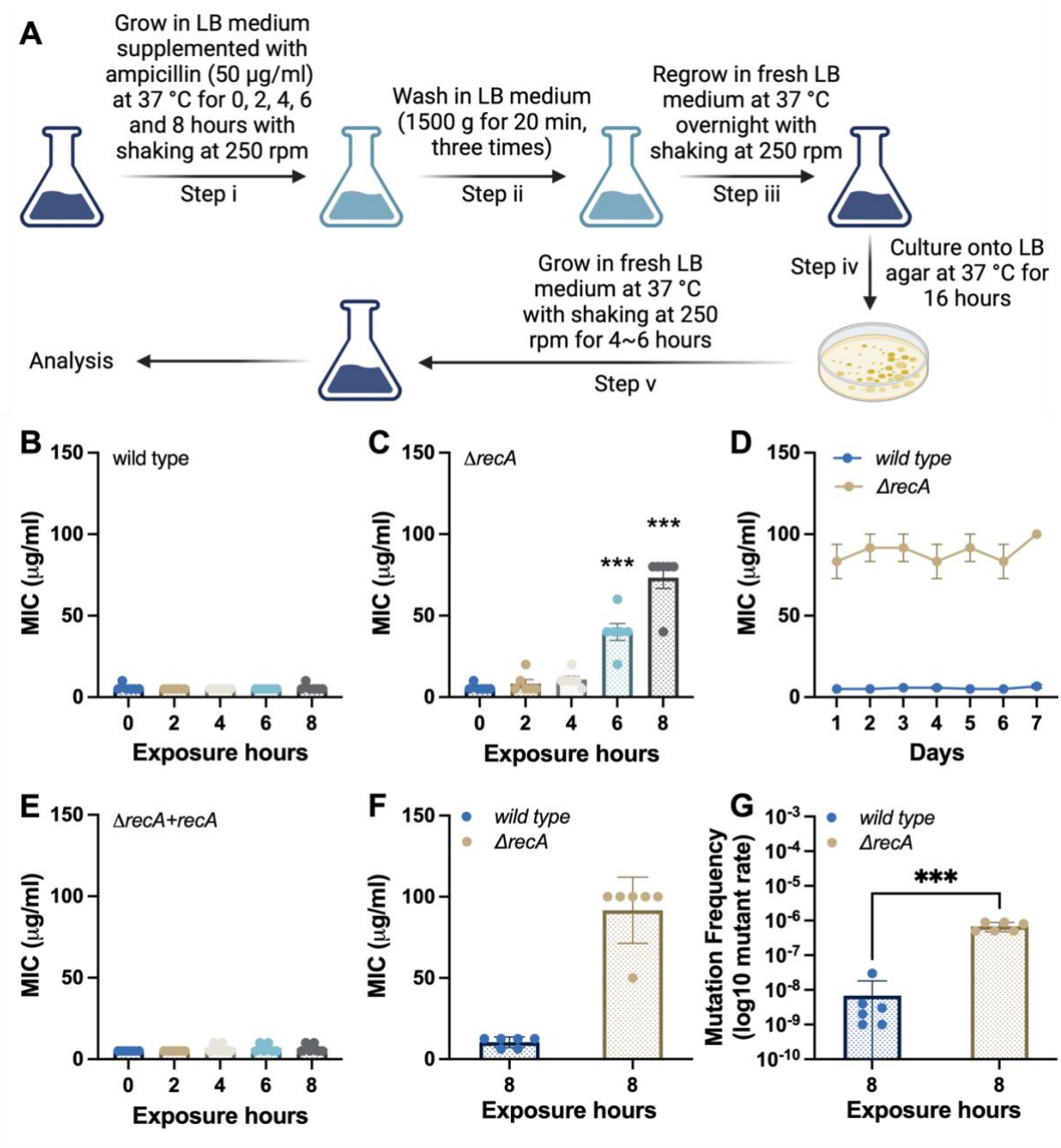


Figure 1. Fast evolution of antibiotic resistance in *E. coli recA* mutant strain. (A) Experimental flow for the single exposures to antibiotics in *E. coli* strains. Step i: an overnight culture (1 x 10^9 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 µg/mL ampicillin and incubated at 37ºC with shaking at 250 rpm for 0, 2, 4, 6 and 8 hours; Step ii: after each treatment, the ampicillin containing medium was removed by washing twice in a fresh LB medium; Step iii: the surviving isolates were resuspended in 30 mL fresh LB medium and regrown overnight at 37ºC with shaking at 250 rpm; Step iv: cell cultures were plated onto LB agar and incubated for 16 hours at 37ºC; Step v: single colonies were inoculated in 30 mL fresh LB medium and cultured at 37ºC with shaking at 250 rpm for 4 to 6 hours. **(B)** MICs of ampicillin were measured against the wild type *E coli* strain after single exposures to

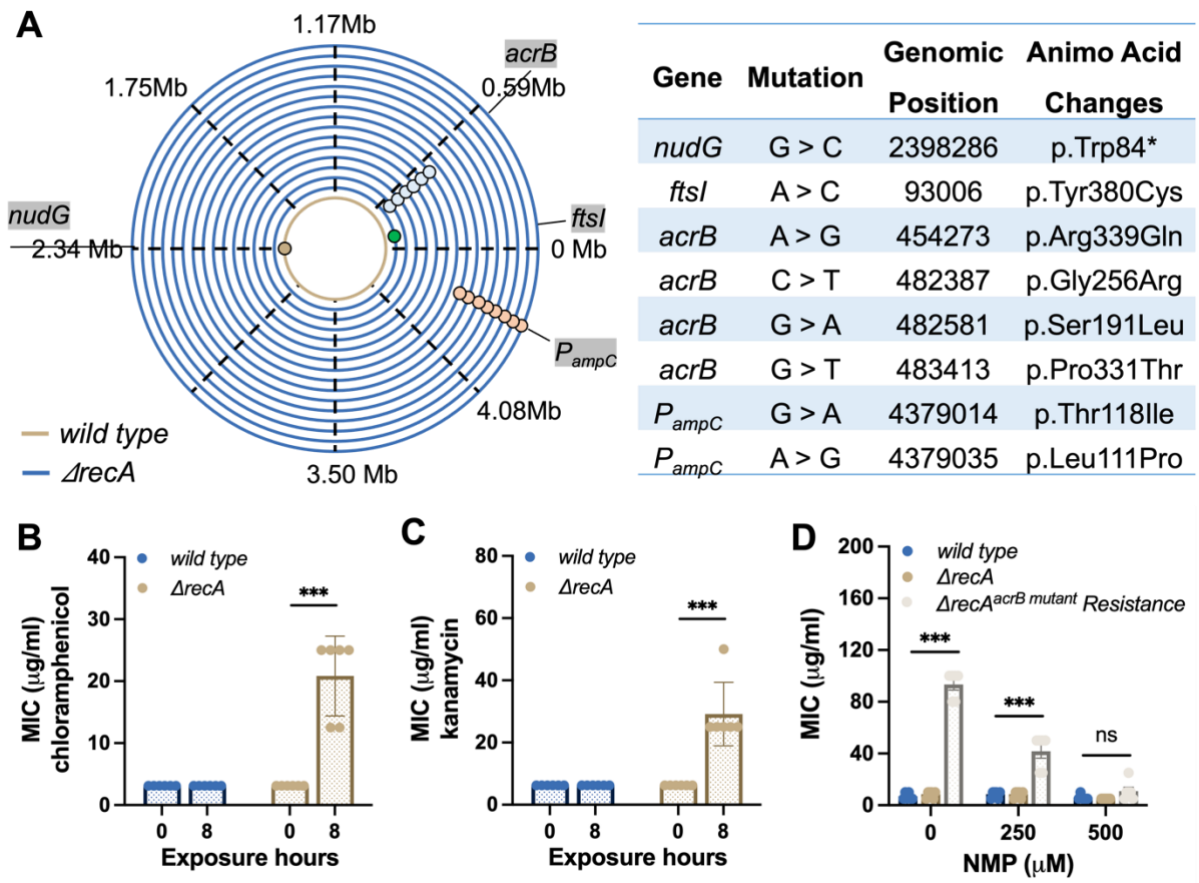
ampicillin. **(C)** MICs of ampicillin were measured against the *ΔrecA* strain after single exposures to ampicillin. **(D)** After the treatment of Step v, cells were continuously cultured in an antibiotic-free medium for seven days. MICs of ampicillin were measured each day. **(E)** MICs of ampicillin were measured against the *ΔrecA* strain treated with ampicillin, where the expression of RecA was restored using plasmid-based constitutive expression of *recA* before the treatment of Step i. **(F)** MICs of rifampicin were measured after an 8-hour treatment of ampicillin for the wild type and *ΔrecA* strain. **(G)** Mutation frequencies using rifampicin as the selector were measured and calculated in the wild type and *ΔrecA* strain after the single exposure to ampicillin. Each experiment was independently repeated at least six times using parallel replicates, and the data are shown as mean ± SEM. Significant differences among different treatment groups are analyzed by independent t-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Rapid appearance of drug resistance associated DNA mutations in *recA* mutant resistant isolates

In bacteria, resistance to most antibiotics requires the accumulation of drug resistance associated DNA mutations developed over time to provide high levels of resistance (29). To verify whether drug resistance associated DNA mutations have led to the rapid development of antibiotic resistance in *recA* mutant strain, we randomly selected 15 colonies on non-selected LB agar plates from the wild type surviving isolates, and antibiotic screening plates containing 50 μg/mL ampicillin from the *ΔrecA* resistant isolates, respectively, and performed whole-genome sequencing. We found that abundant drug resistance associated mutations occurred within all resistant isolates, including the promoter of the β-lactamase *ampC* (P_{ampC}) in 8 isolates, the ampicillin-binding target PBP3 (*ftsI*) in 1 isolate, and the AcrAB-TolC subunit AcrB (*acrB*) mutations in 6 isolates (Fig. 2A). A mutation in gene *nudG* was detected in wild type surviving isolates after the single exposure to ampicillin (Fig. 2A), which is involved in pyrimidine (d)NTP hydrolysis to avoid DNA damage (30). Other mutations were listed in the Table S1.

The presence of P_{ampC} mutations was accompanied by a significant increase in the production of β-lactamase in bacteria (Fig. S4). This leads to specific resistance to β-lactam antibiotics. The gene *acrB* codes for a sub-component of the AcrAB-TolC multi-drug efflux pump, which is central in Gram-negative bacteria (28,31). Mutations in AcrAB-TolC enhance the efflux of

antibiotics and confer resistance to multiple drugs (28). Consequently, after short-term exposure to ampicillin, the *ΔrecA* isolates carrying the *acrB* mutations exhibited resistance to other types of antibiotics, such as chloramphenicol and kanamycin (Fig. 2B and C). Treatment with high concentrations of 1-(1-Naphthylmethyl) piperazine (NMP), an efflux pump inhibitor (EPI) that competitively blocks TolC-composed efflux pumps, successfully restored the sensitivity of *ΔrecA* resistant isolates to ampicillin, bringing them back to the equivalent concentrations found in the wild type (Fig. 2D). Collectively, these results suggest that a single exposure to β-lactam antibiotics can lead to drug resistance associated DNA mutations that play a crucial role in the accelerated development of antibiotic resistance in the *ΔrecA* strain.



Gene	Mutation	Genomic Position	Animo Acid Changes
nudG	G > C	2398286	p.Trp84*
ftsI	A > C	93006	p.Tyr380Cys
acrB	A > G	454273	p.Arg339Gln
acrB	C > T	482387	p.Gly256Arg
acrB	G > A	482581	p.Ser191Leu
acrB	G > T	483413	p.Pro331Thr
P_{ampC}	G > A	4379014	p.Thr118Ile
P_{ampC}	A > G	4379035	p.Leu111Pro

Figure 2. Rapid induction of drug resistance associated DNA mutations in the *recA* mutant strain. (A) Detection of drug resistance associated DNA mutations in the wild type and *ΔrecA* strain after the single exposures to ampicillin at 50 μg/mL for 8 hours. **(B)** MICs of chloramphenicol against the wild type and *ΔrecA* strain after the single treatment with ampicillin were tested. **(C)** MICs of kanamycin against the wild type and *ΔrecA* strain after the single treatment with ampicillin were tested. **(D)** The wild type, *ΔrecA*, and *ΔrecA*$^{acrB\ mutant}$

resistant isolates were incubated with NMP at different concentrations for 12 hours. Subsequently, MICs were tested in these strains for resistance to ampicillin. Each experiment was independently repeated at least six times, and the data is shown as mean ± SEM. Significant differences among different treatment groups are analyzed by independent t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *ns*, no significance.

Hindrance of SOS-independent DNA repair in *recA* mutant resistant isolates

The impaired ability to repair DNA damage can lead to an accelerated accumulation of DNA mutations. The cellular response to DNA damage, such as the SOS response, often influences the balance between bacterial susceptibility and resistance. We thereafter asked whether the absence of *recA* and subsequently induced inhibition of the SOS response can contribute to the rapid development of antibiotic resistance.

To investigate this, we first tested the ability of various mutants involved in different pathways of the SOS response to evolving antibiotic resistance following a single treatment with ampicillin for 8 hours at 50 μg/mL. A mutant form of the SOS master regulator LexA (*lexA3*), which is incapable of being cleaved and thus defective in SOS induction, did not exhibit antibiotic resistance evolution (Fig. 3A). Additionally, the deletion of either DpiB or DpiA (encoded by *citB* or *citA*, respectively), inhibiting the DpiBA two-component signal transduction system, did not result in the development of antibiotic resistance after ampicillin exposure (Fig. 3A). Moreover, the deletion of several downstream effectors of the SOS response, including those involved in cell division inhibition (SulA and YmfM encoded by *sulA* and *ymfM*) (32) (Fig. 3A), and DNA repair (DNA Pol II, DNA Pol IV, and DNA Pol V encoded by *polB*, *dinB* and *umuDC*) (33) also did not lead to the evolution of antibiotic resistance (Fig. 3A). Interestingly, these findings indicate that the fast evolution of antibiotic resistance is supposed to occur in an SOS-independent manner in the absence of *recA*.

In addition to the DNA repair components associated with the SOS response, DNA Pol I plays a role in processing RNA primers during lagging-strand synthesis and filling small gaps during DNA repair reactions (34). Since DNA Pol I (encoded by *polA*) has been demonstrated as an essential gene required for the growth of *E. coli* in rich medium, including the LB medium (35,36), we next utilized Single Molecule Localization Microscopy (SMLM) to precisely locate the chromosome and DNA Pol I in a dynamic manner. During an 8 hour exposure to ampicillin,

we observed the formation of multinucleated filaments in both the wild type and *ΔrecA* strain, indicating a pause in cell division and suggesting a time period for bacterial DNA repair to take place (Fig. 3B and C) (37). However, the expression level of DNA Pol I was significantly suppressed in the *ΔrecA* strain compared to the wild type strain after 4 hours of ampicillin exposure (Fig. 3D). More notably, the super-resolution colocalization analysis revealed a significantly lower ratio of co-localization between the chromosome and DNA Pol I in the *ΔrecA* strain (Fig. 3E), which together demonstrate that the hindrance of DNA repair caused by the absence of *recA* is crucial for the fast evolution of antibiotic resistance in an SOS-independent manner.

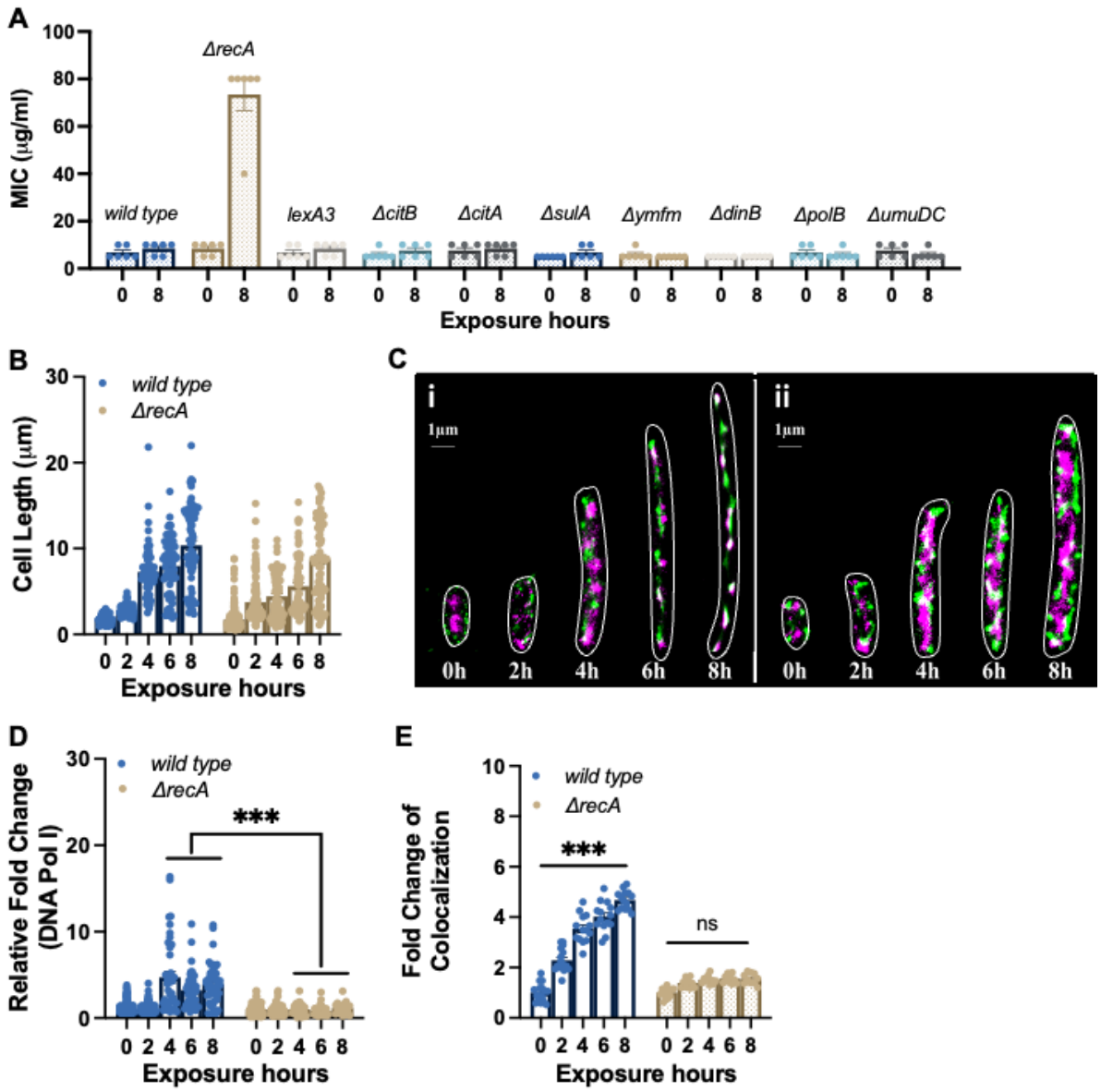


Figure 3. SOS-independent impairment of DNA repair in *ΔrecA* resistant isolates. (A) MICs of ampicillin were tested against the wild type strain, *ΔrecA* strain, and mutants lacking specific genes from the SOS response after single exposures to ampicillin at 50 μg/mL for 8 hours. **(B)** Filament cell lengths in the wild type (*n=253*) and the *ΔrecA* strain (*n=216*) after single treatments with ampicillin at 50 μg/mL. **(C)** Multinucleated filaments were observed in the wild type (i) and the *ΔrecA* (ii) strain after single exposures to ampicillin at 50 μg/mL. Purple: *E. coli* chromosome; green: DNA Pol I. **(D)** Relative fold changes of DNA Pol I in the wild type and *ΔrecA* strain after single treatments with ampicillin at 50 μg/mL. **(E)** Co-localization between the *E. coli* chromosome and DNA Pol I in the wild type and *ΔrecA* strain after the single exposures to ampicillin at 50 μg/mL. Data is shown as mean ± SEM. Significant differences among different treatment groups are analyzed by independent t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *ns*, no significance.

Repression on antioxidative-related gene transcription drives the fast evolution of antibiotic resistance in the *recA* mutant strain

To further comprehend the fast evolution of β-lactam resistance that was observed in this study, we investigated the changes in gene transcription induced by ampicillin treatment using a comprehensive transcriptome sequencing approach (total RNA-seq). Our analysis revealed significant alterations in the transcriptomic profiles of both the wild type and *ΔrecA* strain isolates following a single treatment with ampicillin, compared to untreated controls (Fig. 4A). Specifically, we identified changes in the expression of 161 and 248 coding sequences (with $\log_2 FC > 2$ and P value < 0.05) in the wild type and *ΔrecA* strains, respectively. Principal component analysis (PCA) demonstrated a notable disparity in the effects of ampicillin on the *ΔrecA* strain compared to the wild type strain (Fig. 4B). Additionally, a Venn diagram analysis confirmed that 138 and 225 genes were uniquely regulated by ampicillin exposure in the wild type and *ΔrecA* strains, respectively (Fig. 4C).

To elucidate the differential expression of genes associated with specific biological functions, we conducted Gene Ontology (GO) enrichment analyses (Fig. S5A and B) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (Fig. S5C and D). Our findings indicate that ampicillin profoundly impacted persistence pathways in the wild type strain, specifically affecting pathways related to quorum sensing, flagellar assembly, biofilm formation, and bacterial chemotaxis (38,39). Conversely, in the *ΔrecA* strain, a distinct

functional category associated with the oxidative stress response exhibited significant and unique down-regulation. This category included activities such as sulfate transporter activity, iron-sulfur cluster assembly, oxidoreductase activity, and carboxylate reductase activity.

To identify specific genes showing significant fold changes ($\log_2 FC > 2$ and P value < 0.05), we used volcano plots to visualize the comprehensive changes in gene expression across the genome (Fig. 4D). We examined the transcription levels associated with the SOS response system and found that the transcription of several proteins in the wild type strain can be significantly induced by the single exposure to ampicillin, including *citB* and *dinB* (Fig. 4E). However, in the *recA* mutant strain, antibiotic exposure does not affect the transcription levels of any SOS system-related proteins, suggesting that antibiotic exposure induced the SOS response in the wild type strain but not in the *ΔrecA* strain. More importantly, we discovered that the induction of the transcription level of the DNA Pol I was significantly suppressed after the single treatment of ampicillin in the *ΔrecA* strain compared with that in the wild type strain (Fig. 4E). This is consistent with our imaging results and further supports the notion that an SOS-independent evolutionary mechanism dominates the development of antibiotic resistance in *recA* mutant *E. coli*.

Further, significant downregulation in the transcription of antioxidative-related genes in the *ΔrecA* strain was detected, including *cysJ*, *cysI*, *cysH*, *soda*, and *sufD* (Fig. 4F). This downregulation suggested an excessive accumulation of reactive oxygen species (ROS) due to compromised cell antioxidative defences. It has been previously reported that the induction of mutagenesis can be stimulated by the overproduction of ROS during antibiotic administration, leading to the evolution of antibiotic resistance both *in vivo* and *in vitro* (22,23). Therefore, we hypothesized that elevated ROS levels might be responsible for the observed fast evolution of antibiotic resistance in the *ΔrecA* strain. To test it, we supplemented the wild type and *ΔrecA* strains with 50 mM glutathione (GSH), a natural antioxidative compound, and treated them with ampicillin at a concentration of 50 μg/ml for 8 hours. Remarkably, the addition of GSH prevented the development of resistance to ampicillin in the *ΔrecA* strain (Fig. 4G), without impairing the bactericidal effectiveness of ampicillin (Fig. 4H).

Apart from the SOS response, bacterial cells coordinate other DNA repair activities through a network of regulatory pathways, including excision repair (BER) (40-42). The excessive generation of ROS results in elevated levels of deoxy-8-oxo-guanosine triphosphate (8-oxo-

dGTP), an oxidized form of dGTP that becomes both highly toxic and mutagenic upon integration into DNA. The presence of 8-oxo-dG can induce SNP mutations, especially those occurring in guanine, which can be actively rectified by the BER repair pathway (42). Notably, BER glycosylases MutH and MutY can identify and repair these 8-oxo-dG-dependent mutations; however, when MutY and MutH are inactivated, unrepaired 8-oxo-dG can lead to the accumulation of SNP mutations within cells (42). As a result, we conducted a further assessment of the transcription levels of the BER repair pathway in both the wild-type and *ΔrecA* strain before and after a single exposure to ampicillin. We discovered that after an 8-hour treatment of ampicillin, three DNA repair-associated proteins, including MutH, MutY, and MutM, were notably suppressed in the *ΔrecA* strain (Fig. 4I).

Finally, we sequenced the surviving *ΔrecA* isolates and found that the addition of GSH inhibited drug resistance associated mutations in the *ΔrecA* strain, which was detected in the *ΔrecA* resistant isolates including genes of the promoter of *ampC* and *acrB* (Fig. 4J). Given the DNA repair impairment resulting in the generation of ROS, it would have been expected for genes involved in the oxidative stress response to be induced in RecA-deficient cells. However, the repression of antioxidative-related genes indicated the involvement of transcriptional repressors that might be regulated by RecA. Consequently, we examined the transcription levels of all transcriptional repressors in both the wild type and *ΔrecA* strain. Remarkably, we observed a significant upregulation of H-NS, a crucial transcriptional repressor (Fig. 4K). This finding suggests that the upregulation of H-NS could potentially account for the downregulation of antioxidant gene transcription levels in the *ΔrecA* strain.

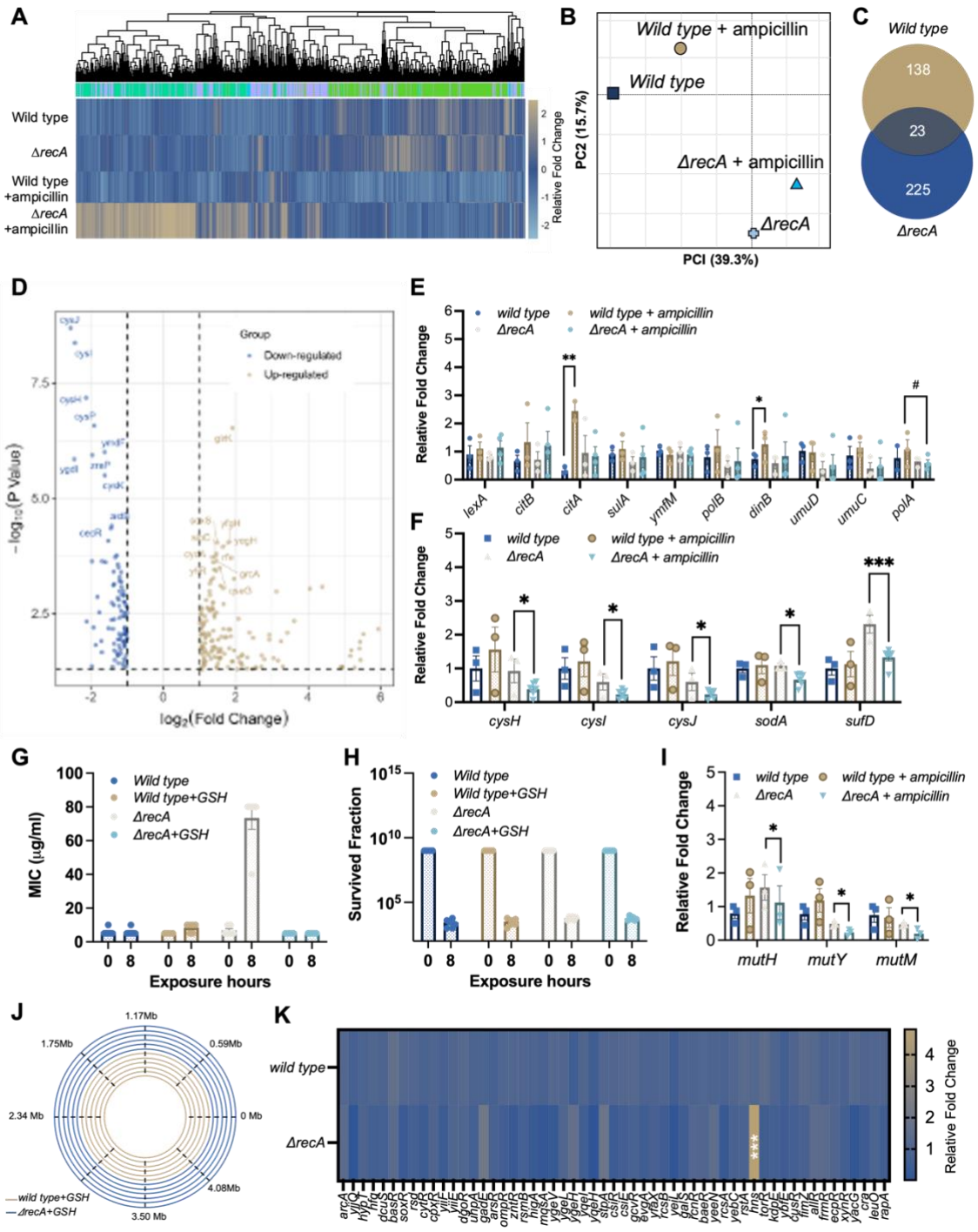


Figure 4. Overaccumulation of ROS drives the fast evolution of multi-drug resistance in the *ΔrecA* strain. (A) Clustered heatmap of relative expression of coding sequences in the wild type and *ΔrecA* strain with significant fold changes ($\log_2$FC > 2 and *P* value < 0.05). **(B)** Principal-component analysis (PCA) of normalised read counts for all strains. **(C)** Venn diagram of differentially expressed genes ($\log_2$FC > 2) after treatment with ampicillin at 50 μg/ml for 8 hours in the wild type and *ΔrecA* strain. **(D)** The top 10 most differentially expressed genes in the *ΔrecA* strain after the single treatment with ampicillin are labelled in

each plot. Blue dots indicate genes with a significant downregulation compared to the untreated control ($\log_2$FC > 2 and *P* value < 0.05), and yellow dots indicate genes with a significant upregulation compared to the untreated control ($\log_2$FC > 2 and *P* value < 0.05). **(E)** Levels of transcription of SOS response system-associated genes and gene *polA* in the wild type and *ΔrecA* strain after single exposures to ampicillin for 8 hours. **(F)** Levels of transcription of different antioxidative associated genes in the wild type and *ΔrecA* strain after single exposures to ampicillin for 8 hours. **(G)** The addition of 50 mM antioxidative compound GSH prevented the evolution of antibiotic resistance to ampicillin in the *ΔrecA* strain treated with ampicillin at 50 μg/ml for 8 hours. **(H)** Survival fraction after a single exposure to ampicillin at 50 μg/ml for 8 hours in the wild type and the *ΔrecA* strain with or without the addition of GSH at 50 mM. **(I)** Levels of transcription of proteins involved in the BER DNA repair system in the wild type and *ΔrecA* strain after single exposures to ampicillin for 8 hours. **(J)** Whole genome sequencing confirms undetectable DNA mutations in the wild type and *ΔrecA* strain treated with single exposures to ampicillin with the addition of GSH at 50 mM for 8 hours. **(K)** Transcription levels of all transcriptional repressors in the wild type and *ΔrecA* strain after single treatments with ampicillin for 8 hours. Total RNA-seq was performed with three repeats in each group. Each experiment was independently repeated at least six times, and the data are shown as mean ± SEM. Significant differences among different treatment groups are analyzed by independent t-test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, #*P* < 0.05.

Discussion

In this study, we present a novel perspective challenging the conventional belief that disabling RecA to inhibit the SOS response can prevent bacteria from developing antibiotic resistance (10). While the SOS response and RecA have been extensively studied for their roles in antibiotic resistance evolution (43), we observed a fast evolution of multi-drug resistance in the *ΔrecA* strain triggered by single exposures to β-lactam antibiotics, which was driven by three mechanisms, including the impaired DNA repair, transcriptional repression of antioxidative-related genes, and excessive accumulation of ROS (Fig. 5).

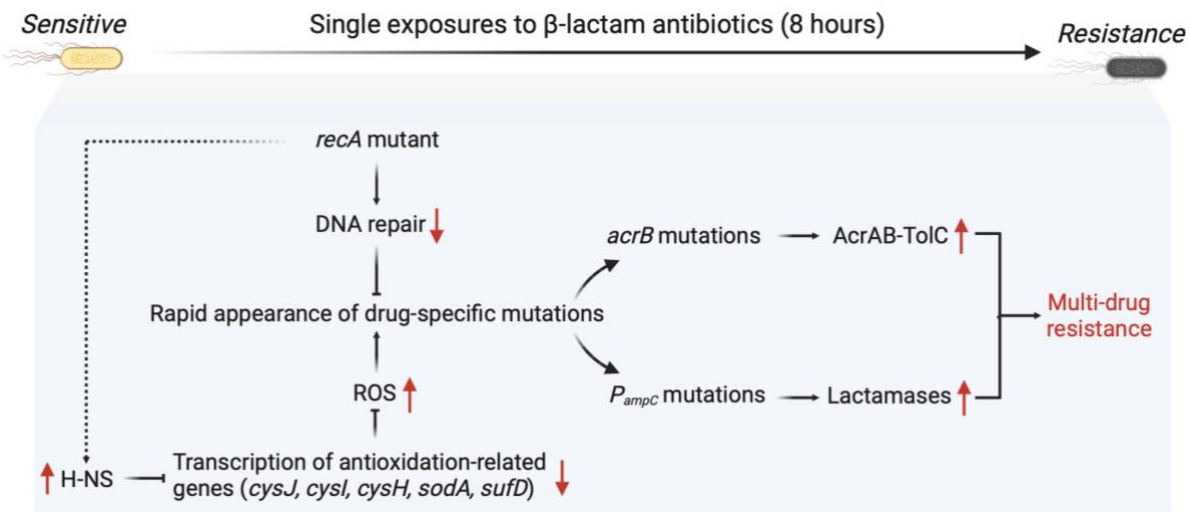


Figure 5. Mechanism of rapid development of multi-drug resistance in the *recA* mutant *E. coli* strain. The deletion of *recA* results in a decrease in DNA damage repair capability. Additionally, the absence of *recA* upregulates the transcription levels of the transcriptional repressor H-NS, although the mechanism of this regulation is still unclear. Upregulation of H-NS potentially inhibits the transcription levels of several downstream genes associated with antioxidant functions, leading to an excessive accumulation of ROS. The excessive production of ROS causes an increased occurrence of genetic mutations. Under antibiotic pressure, mutations related to drug resistance are selected, ultimately leading to the emergence of rapid multidrug resistance.

We initially suspected that the decrease in DNA repair levels was caused by the suppression of the SOS response, mediated by the deficiency of RecA during rapid resistance development. We therefore examined the response to antibiotic exposure in various *E. coli* mutants lacking specific genes from the SOS response. However, we discovered that these strains remained susceptible after short-term exposure to ampicillin, providing evidence that the decrease in DNA repair levels, regulated by RecA in an SOS-independent manner, served as a mechanism for the accelerated development of antibiotic resistance. Furthermore, we conducted super-resolution imaging studies to analyze the expression and localization of DNA Pol I. We observed a significant inhibition of DNA Pol I induction and its involvement in DNA repair. Moreover, a notable suppression of the BER repair system was observed in the *recA* mutant bacteria upon short-term exposure to antibiotics. While we do not yet have evidence explaining why the absence of *recA* might affect the transcriptional regulation of the BER repair system, this evidence is sufficient to demonstrate that the absence of RecA can lead to the accumulation

of beneficial mutations within bacteria through downregulation of DNA repair capacity. The regulation of DNA repair by RecA is a significantly complex process (44), and although we specifically demonstrated the regulation of DNA Pol I and BER repair system by RecA under antibiotic exposure, there should be other repair mechanisms impaired and contributed to this fast evolution of resistance, which needs to be investigated in future.

Antibiotics exert their bactericidal effects by targeting specific intracellular components, and the production of ROS is considered an alternative mechanism underlying quinolone-induced bacterial killing. However, emerging evidence suggests that lethality may arise from primary antibiotic-induced damage or a secondary response to lethal stress mediated by ROS. ROS generation can also contribute to the development of multidrug resistance, as it can directly damage DNA, leading to genetic mutations. In this study, we demonstrate that excessive ROS accumulation drives the evolution of antibiotic resistance in bacteria with impaired DNA repair capabilities. Significantly, we discovered for the first time that antibiotic exposure can significantly increase the transcription of H-NS in the absence of *recA*. *E. coli* H-NS is a DNA-binding protein known for its involvement in transcriptional repression (45). It binds to DNA in a sequence-independent manner, with a preference for A-/T-rich regions in curved DNA, thereby impeding transcription (46). Previous findings report that H-NS can down-regulate the transcriptional expression of *cysIJH* through the mediation of *cysB* (47,48). While we have established a potential correlation between the increased transcription of H-NS genes induced by antibiotic exposure in RecA deficient cells and the decreased transcription of genes related to antioxidative processes, further investigations are imperative to elucidate the regulatory mechanisms employed by RecA in H-NS transcription and its inhibitory effects on antioxidative gene transcription.

In clinics, the resistance mechanism to β-lactams is not only associated with β-lactamase acquisition but also with increased expression of efflux pumps (44). Our research for the first time demonstrates the fast evolution of antibiotic resistance through the rapid accumulation of *acrB* mutations and enhanced functionality of the AcrAB-TolC efflux pump in RecA-deficient bacteria exposed to a single dose of ampicillin. This finding supports the clinical observations that the clinical antibiotic resistance is accelerating (50).

DNA repair is a key target for many anti-cancer drugs that aim to destroy tumor cells by inducing DNA damage (51). Homologous recombination (HR) plays a crucial role in DNA

repair, and Rad51, a vital protein in the HR pathway of eukaryotic cells, belongs to the *recA*/RAD51 gene family (51-53). Clinical trials are currently assessing a range of Rad51 inhibitors. Interestingly, studies have revealed that inhibitors targeting Rad51 can effectively inhibit the activity of RecA (54). Despite RecA and Rad51 sharing only approximately 30% sequence homology, the filaments they form and the conformational changes they induce in DNA are remarkably similar (54). Notably, inhibitors of RecA have demonstrated the ability to enhance the antimicrobial effects of certain antibiotics, including fluoroquinolones (55,56). Our findings have significant clinical implications, especially for patients undergoing cancer treatment. The combination therapy involving β-lactam antibiotics and RecA inhibitors could potentially accelerate the evolution of multi-drug resistance at an alarming rate.

Our findings on the mechanism of the fast evolution of multidrug resistance in *E. coli* after a single exposure to a specific antibiotic can be applied more broadly. The overproduction of ROS is a common effect of many antibiotics and other strategies used to combat infections, such as antimicrobial photodynamic therapy (aPDT) and cold atmospheric plasma (CAP) (57,58). These approaches rely on ROS-mediated damage as the primary source of stress-induced damage. Thus, maintaining the transcription of genes involved in the oxidative stress response or the combined treatment with drugs and antioxidants shows promising potential to prevent stress-induced mutagenesis that is typically triggered by various classes of antibiotics.

Acknowledgements

This work was supported by the Australian Research Council (ARC grant no: APP1165135), Science and Technology Innovation Commission of Shenzhen (KQTD20170810110913065), Australia China Science and Research Fund Joint Research Centre for Point-of-Care Testing (ACSRF658277, SQ2017YFGH001190).

Author contributions

L.Z., G.P., Q.S., A.R., E.H., A.O. and D.J. designed the experiments. L.Z., N.C., A.B., E.H., and D.J. wrote the manuscript with input from all co-authors. L.Z., G.P., Q.S., and Y.Y.C. conducted experiments and analysed data.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All data are available in the main text or the supplementary materials.

Reference

1. Rebecca S. Antimicrobial-Induced DNA Damage and Genomic Instability in Microbial Pathogens. *PLOS Pathogens*. **11**, e1004678 (2015).
2. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*. **7**, 130 (5): 797-810 (2007).
3. Baquero F, Levin BR. Proximate and ultimate causes of the bactericidal action of antibiotics. *Nat Rev Microbiol*. **19** (2): 123-132 (2021).
4. Žgur-Bertok D. DNA Damage Repair and Bacterial Pathogens. *PLOS Pathogens.* **9** *PLOS Pathogens*, e1003711 (2013).
5. Dale B. Wigley. Bacterial DNA Repair: Recent Insights into The Mechanism of RecBCD, AddAB and AdnAB. *Nature Reviews Microbiology*. **11**, 9-13 (2013).
6. Harms A, Maisonneuve E, Gerdes K. Mechanisms of Bacterial Persistence during Stress and Antibiotic Exposure. *Science*. **354**, aaf4268 (2016).
7. Katarzyna H. Maslowska, Karolina Makiela-Dzbenska, Iwona J. Fijalkowska. The SOS System: A Complex and Tightly Regulated Response to DNA Damage. *Environ Mol Mutagen.* **60**, 368-384 (2019).
8. Michael M. Cox. Motoring Along with the Bacterial RecA Protein. *Nature Reviews Molecular Cell Biology*. **8**, 127-138 (2007).
9. Barrett, T.C., Mok, W.W.K., Murawski, A.M. et al. Enhanced antibiotic resistance development from fluoroquinolone persisters after a single exposure to antibiotic. *Nat Commun*. **10**, 1177 (2019).
10. Athanasia Pavlopoulou. RecA: A Universal Drug Target in Pathogenic Bacteria. *Frontiers in Bioscience*. **23**, 36-42 (2018).
11. Mercolino J, Lo Sciuto A, Spinnato MC, Rampioni G, Imperi F. RecA and Specialized Error-Prone DNA Polymerases Are Not Required for Mutagenesis and Antibiotic Resistance Induced by Fluoroquinolones in Pseudomonas aeruginosa. *Antibiotics (Basel)*. **11**, 3, 325 (2022).
12. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN. SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science*. **305** (5690), 1629-31 (2004).

13. Jahn LJ, Munck C, Ellabaan MMH, Sommer MOA. Adaptive Laboratory Evolution of Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and Genotypes. *Front Microbiol*. **8**:816 (2017).

14. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. Antibiotic Tolerance Facilitates the Evolution of Resistance. *Science*. **355**, 826-830 (2017).

15. Dwyer DJ, Kohanski MA, Hayete B, Collins JJ. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol Syst Biol*. **3**: 91 (2007).

16. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*. **7**, 130(5): 797-810 (2007).

17. Luan, G, Hong, Y, Drlica, K, and Zhao, X. Suppression of reactive oxygen species accumulation accounts for paradoxical bacterial survival at high quinolone concentration. *Antimicrob. Agents Chemother*. 62: e01617-e01622 (2018).

18. Zhao X, Drlica K. Reactive oxygen species and the bacterial response to lethal stress. *Curr Opin Microbiol.* **21**, 1-6 (2014).

19. Zhao X, Hong Y, Drlica K. Moving forward with reactive oxygen species involvement in antimicrobial lethality. *J Antimicrob Chemother*. **70** (3): 639-642 (2015).

20. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science*. **339** (6124):1213-1216 (2013).

21. Imlay JA. Diagnosing oxidative stress in bacteria: not as easy as you might think. *Curr Opin Microbiol*. **24**, 124-131 (2015).

22. Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol Cell*. **37**(3): 311-320 (2010).

23. Takahashi N, Gruber CC, Yang JH, Liu X, Braff D, Yashaswini CN, Bhubhanil S, Furuta Y, Andreescu S, Collins JJ, Walker GC. Lethality of MalE-LacZ hybrid protein shares mechanistic attributes with oxidative component of antibiotic lethality. *Proc Natl Acad Sci USA*. **114** (34): 9164-9169 (2017).

24. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol*. **12**, 465-478 (2014).

25. Fridman, O., Goldberg, A., Ronin, I. et al. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*, **513**, 418-421 (2014).

26. Reimer LG, Stratton CW, Reller LB. Minimum inhibitory and bactericidal concentrations of 44 antimicrobial agents against three standard control strains in broth with and without human serum. *Antimicrob Agents Chemother*., **19**, 1050-1055 (1981).

27. Jones RN, Fuchs PC, Gerlach EH, Gavan TL. Piperacillin and carbenicillin; a collaborative in vitro comparison against 10,838 clinical bacterial isolates. *Cleveland Clinic Journal of Medicine*, **46**, 49-55 (1979).

28. El Meouche I, Dunlop MJ.. Heterogeneity in Efflux Pump Expression Predisposes Antibiotic-Resistant Cells to Mutation. *Science*. **362**, 686-690 (2018).

29. Toprak E, Veres A, Michel JB, Chait R, Hartl DL, Kishony R. Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat Genet*. **44** (1), 101-105 (2011).

30. Kamiya H, Murata-Kamiya N, Iida E, Harashima H. Hydrolysis of oxidized nucleotides by the Escherichia coli Orf135 protein. *Biochem Biophys Res Commun*. **288** (3): 499-502 (2001).

31. Jessica M. A. Blair et al. Molecular Mechanisms of Antibiotic Resistance. *Nature Reviews Microbiology*. **13**, 42-51 (2015).

32. Simmons LA, Foti JJ, Cohen SE, Walker GC. The SOS regulatory network. *EcoSal Plus*. 5.4.3 (2008).

33. Ansari S, Walsh JC, Bottomley AL, Duggin IG, Burke C, Harry EJ. A newly identified prophage-encoded gene, *ymfM*, causes SOS-inducible filamentation in *Escherichia coli*. *J Bacteriol.* **15**, 203(11): e00646-20 (2015).

34. Monk M, Peacey M, Gross JD. Gross. Repair of Damage Induced By Ultraviolet Light in DNA Polymerase-Defective *Escherichia Coli* Cells. *Journal of Molecular Biology*. **58**, 623-624 (1971).

35. Joyce CM, Grindley ND. Method for determining whether a gene of *Escherichia coli* is essential: application to the *polA* gene. *J Bacteriol*. 158(2): 636-643 (1984).

36. Konrad EB, Lehman IR. A conditional lethal mutant of *Escherichia coli* K12 defective in the 5' leads to 3' exonuclease associated with DNA polymerase I. *Proc Natl Acad Sci*. 71(5): 2048-2051 (1974).

37. Justice SS, Hunstad DA, Cegelski L, Hultgren SJ. Morphological Plasticity as a Bacterial Survival Strategy. *Nature Review Microbiology*. **6**, 162-168 (2008).

38. Helaine S, Kugelberg E. Bacterial Persisters: Formation, Eradication, and Experimental Systems. *Trends in Microbiology*. **22**, 417-424 (2014).

39. Amy L. Spoering, Kim Lewis. Bio¢Lms and Planktonic Cells of *Pseudomonas Aeruginosa* Have Similar Resistance to Killing by Antimicrobials. *J. Bacteriol.* **183**, 6746-6751 (2001).

40. Foti JJ, Devadoss B, Winkler JA, Collins JJ, Walker GC. Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science*. 336(6079): 315-319 (2012).

41. DNA repair and mutagenesis (2nd ed.). *ASM Press* (2005).

42. Hahm JY, Park J, Jang ES, Chi SW. 8-Oxoguanine: from oxidative damage to epigenetic and epitranscriptional modification. *Exp Mol Med*. 54(10): 1626-1642 (2022).

43. Pavlopoulou A. RecA: A Universal Drug Target in Pathogenic Bacteria. *Frontiers in Bioscience*. **23**, 36-42 (2018).

44 Smith KC, Wang TC. *recA*-dependent DNA repair processes. *Bioessays*. **10** (1):12-6 (1989).

45. Dorman CJ. H-NS: a universal regulator for a dynamic genome. *Nat Rev Microbiol*. **2** (5):391-400 (2004).

46. Bracco L, Kotlarz D, Kolb A, Diekmann S, Buc H. Synthetic curved DNA sequences can act as transcriptional activators in Escherichia coli. *EMBO J*. **8** (13):4289-96 (1989).

47. Álvarez R, Neumann G, Frávega J, Díaz F, Tejías C, Collao B, Fuentes JA, Paredes-Sabja D, Calderón IL, Gil F. CysB-dependent upregulation of the Salmonella Typhimurium cysJIH operon in response to antimicrobial compounds that induce oxidative stress. *Biochem Biophys Res Commun*. **458** (1):46-51 (2015).

48. Ishihama A, Shimada T. Hierarchy of transcription factor network in *Escherichia coli* K-12: H-NS-mediated silencing and Anti-silencing by global regulators. FEMS Microbiol Rev. **45** (6):fuab032 (2021).

49. Pages JM, Lavigne JP, Leflon-Guibout V, Marcon E, Bert F, Noussair L, Nicolas-Chanoine MH. Efflux pump, the masked side of beta-lactam resistance in Klebsiella pneumoniae clinical isolates. *PLoS One*. 4(3), e4817 (2009).

50. Witzany C, Bonhoeffer S, Rolff J. Is antimicrobial resistance evolution accelerating? *PLoS Pathog*. 16(10): e1008905 (2020).

51. Li X, Heyer WD. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Research*. **18**, 99-113 (2008).

52. PR Bianco, RB Tracy, SC Kowalczykowski. DNA strand exchange proteins: a biochemical and physical comparison. *Front Biosci.* **3**, D570-603 (1998).

53. P Sung, L Krejci, S Van Komen, MG Sehorn, Rad51 recombinase and recombination mediators. *J Biol Chem.* 278, 42729-42732 (2003).

54. T Ogawa, X Yu, A Shinohara, EH Egelman, Similarity of the yeast RAD51 filament to the bacterial RecA filament. *Science* **259**, 1896-1899 (1993).

55. Wigle TJ, Sexton JZ, Gromova AV, Hadimani MB, Hughes MA, Smith GR, Yeh LA, Singleton SF. Inhibitors of RecA activity discovered by high-throughput screening: cell-permeable small molecules attenuate the SOS response in Escherichia coli. *J Biomol Screen*. **14**(9), 1092-101 (2009).

56. Yakimov A, Pobegalov G, Bakhlanova I, Khodorkovskii M, Petukhov M, Baitin D. Blocking the RecA activity and SOS-response in bacteria with a short α-helical peptide. *Nucleic Acids Res*. **45**(16), 9788-9796 (2017).

57. Wilson, BC, and Patterson, MS. The physics, biophysics and technology of photodynamic therapy. *Phys. Med. Biol*. **53**, R61-R109 (2008).

58. Vatansever F, de Melo WC, Avci P, Vecchio D, Sadasivam M, Gupta A, Chandran R, Karimi M, Parizotto NA, Yin R, Tegos GP, Hamblin MR. Antimicrobial strategies centered around reactive oxygen species-bactericidal antibiotics, photodynamic therapy, and beyond. *FEMS Microbiol Rev*. **37**(6): 955-989 (2013).

59. Ghodke H, Paudel BP, Lewis JS, Jergic S, Gopal K, Romero ZJ, Wood EA, Woodgate R, Cox MM, van Oijen AM. Spatial and temporal organization of RecA in the *Escherichia coli* DNA-damage response. *Elife*, **8**: e42761 (2019).

60. Eric M. Scholar, William B. Pratt. *The Antimicrobial Drugs*. Oxford University Press.

61. A. Gutierrez, L. Laureti, S. Crussard, H. Abida, A. Rodríguez-Rojas, J. Blázquez, Z. Baharoglu, D. Mazel, F. Darfeuille, J. Vogel, I. Matic, β-Lactam antibiotics promote bacterial mutagenesis via a RpoS-mediated reduction in replication fidelity. *Nat. Commun*. 4, 1610 (2013).

62. Kirill A. Datsenko, Barry L. Wanner. One-Step Inactivation of Chromosomal Genes in *Escherichia Coli* K-12 Using PCR Products. *Proc Natl Acad Sci*. **97**, 6640-6645 (2000).

63. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia Coli* K-12 In-Frame, Single-Gene Knockout Mutants: The Keio Collection. *Mol Syst Biol*, **2**, 2006.0008 (2006).

64. Van der Auwera GA & O'Connor BD. Genomics in the Cloud: Using Docker, GATK, and WDL in Terra (1st Edition). *O'Reilly Media*. (2020).

65. Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin)*. **6**, 2, 80-92 (2012)

66. Liao Y, Smyth GK, Shi W. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res*. **30**, 923 (2019).
67. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
68. Risso D, Ngai J, Speed TP, Dudoit S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol* **32**, 896-902 (2014).
69. Su QP, Zhao ZW, Meng L, Ding M, Zhang W, Li Y, Liu M, Li R, Gao YQ, Xie XS, Sun Y. Superresolution Imaging Reveals Spatiotemporal Propagation of Human Replication Foci Mediated By CTCF-Organized Chromatin Structures. *Proceedings of the National Academy of Sciences*. **117**, 15036-15046 (2020).
70. Wang G, Li Y, Wang P, Liang H, Cui M, Zhu M, Guo L, Su Q, Sun Y, McNutt MA, Yin Y. PTEN Regulates RPA1 and Protects DNA Replication Forks. *Cell Res*. **25**, 1189-1204 (2015).
71. Huang B, Wang W, Bates M, Zhuang X. Three-Dimensional Super-Resolution Imaging by Stochastic Optical Reconstruction Microscopy. *Science*. **319**, 810-813 (2008).
72. Levet F, Hosy E, Kechkar A, Butler C, Beghin A, Choquet D, Sibarita JB. SR-tesseler: A Method to Segment and Quantify Localization-Based Super-Resolution Microscopy Data. *Nat. Methods*. **12**, 1065–1071 (2015).

Materials and Methods

Bacterial strains, medium and antibiotics

Bacterial strains and plasmids used in this work are described in Table S2 and Table S3. Luria-Bertani (LB) was used as broth or in agar plates. *E. coli* cells were grown in LB liquid medium or on LB agar (1.5% w/v) plates at 37ºC, unless stated otherwise, antibiotics were supplemented, where appropriate. Antibiotic stock solutions were prepared by dissolving antibiotics in MilliQ filter sterilising, including ampicillin (50 mg/mL), penicillin G (100 mg/mL), carbenicillin (20 mg/mL), kanamycin (50 mg/mL) and tetracycline (10 mg/mL). Chloramphenicol stock solution was prepared in 95% EtOH (25 mg/mL). Antibiotic solutions were stored at -20ºC (long-term) or 4ºC (short-term).

Treatment with antibiotics to induce evolutionary resistance

For the single exposure to antibiotic experiment, an overnight culture (0.6 mL; 1 x 10^9 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with antibiotics (50 μg/mL

ampicillin, 1 mg/mL penicillin G, or 200 μg/mL carbenicillin) and incubated at 37ºC with shaking at 250 rpm for 0, 2, 4, 6 and 8 hours, respectively. After each treatment, the antibiotic-containing medium was removed by washing twice (20 min centrifugation at 1500 g) in fresh LB medium (See Fig. 1A for method overview).

To test resistance, the surviving isolates were first resuspended in 30 mL LB medium and grown overnight at 37ºC with shaking at 250 rpm. The regrown culture was then plated onto LB agar and incubated overnight at 37ºC. Single colonies were isolated and grown in LB medium for 4-6 hours at 37ºC with shaking at 250 rpm, which were then used to test the resistance or stored at -80ºC for future use.

For the ALE antibiotic treatment experiments, an overnight culture (0.6 mL; 1 x 10^9 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 μg/mL ampicillin and incubated at 37ºC with shaking at 250 rpm for 4 or 8 hours. After treatment, the antibiotic-containing medium was removed by washing twice (20 min centrifugation at 1500 g) in a fresh LB medium. The remaining pellet was resuspended in 30 mL LB medium and grown overnight at 37ºC with shaking at 250 rpm. Ampicillin treatment was applied to the regrown culture and repeated until resistance was established, as confirmed by MIC measurement.

Antibiotic susceptibility testing

The susceptibility of *E. coli* cells to antibiotics was measured using minimum inhibitory concentration (MIC) testing (60). In brief, overnight cultures were diluted and incubated at 37ºC for 4-6 hours with shanking at 250 rpm. Cells were then diluted 1:100 and incubated with increasing concentrations of antibiotics in the Synergy HT BioTek plate reader (BioTek Instruments Inc., USA) at 37ºC for 16 hours. It was programmed to measure the OD hourly at 595 nm (Gen5 software, BioTek Instruments Inc., USA). The minimum inhibitory concentration was determined as the concentration of antibiotic where no visible growth was observed.

Mutation frequency

Overnight cultures inoculated from single colonies in LB medium were diluted 1:1,000,000 and incubated at 37°C with shaking until the OD_{600} reached to 1~1.3. This extreme dilution minimizes the presence of pre-existing stationary phase mutants. The total number of colony-forming units per ml (CFU/ml) was determined by plating on LB agar. To count mutants, cells

were centrifuged and plated on LB agar with an appropriate concentration of antibiotic. LB plates were incubated for 24 hours at 37°C and selective plates were incubated for 48-72 hours at 37°C (61). The mutation frequency was then determined as the CFU/ml on LB + selective antibiotic agar plates divided by the CFU/ml on LB agar plates.

Construction of *recA* deletion mutant

Lambda Red recombination was used to generate the gene *recA* deletion in the *E. coli* K-12 strain, followed by previously reported methods with modifications (62,63). Primers (*recA-FWD* and *recA-REV*, Table S4) were designed approximately 50 bp upstream and downstream to the gene *recA* on the chromosome to amplify the tetracycline cassette as well as the flanking DNA sequence needed for homologous recombination. Phusion polymerase (NEB) was used to amplify the DNA sequence (Table S4), and the reaction was cleaned up using a PureLink™ PCR purification kit (ThermoFisher Scientific) as per the manufacturer's instructions. Electro-competent *E. coli* MG1655 containing the recombinase plasmid pKD46 was transformed with 50 ng of amplified DNA a 30°C. The transformation was plated onto LB agar plates containing 10 μg/mL tetracycline and incubated overnight at 37°C. PCR was used to confirm the insertion of the tetracycline resistance cassette at the correct site on the chromosome using primers upstream and downstream to the gene *recA*. The newly constructed mutant strains were cured of plasmid pKD46 by incubating LB streak plates at 42°C overnight. Loss of the plasmid was confirmed by lack of ampicillin sensitivity on LB agar plates. Mutant strains were made electro-competent, and 50 μL of cells were transformed with plasmid pCP20 and incubated on 100 μg/mL ampicillin plates at 30°C overnight. A few colonies were then restreaked onto LB plates and incubated overnight at 42°C. PCR products confirmed the loss of cassette and plasmid.

β-lactamase assay

The amount of β-lactamase was measured using a β-lactamase Activity Assay Kit (Sigma-Aldrich, US). Briefly, cells were collected by centrifugation at 10,000 g for 10 min, and the pellet was resuspended with 5 μL of assay buffer per mg of sample. Then, 48 μL of the sample was mixed with 2 μL of nitrocefin. The β-Lactamase activity was monitored by measuring the absorbance at 490 nm for 30 min at 28°C. The level of β-lactamase was determined by the absorbance at OD_{390}.

Whole genome sequencing

Resistant clones were isolated by selection using LB agar plates with the supplementation of ampicillin at 50 μg/mL. Chromosomal DNA was extracted and purified using the PureLink™ Genomic DNA mini kit following the manufacturer's instructions (ThermoFisher Scientific). Whole genome sequencing (WGS) was conducted following the Nextera Flex library preparation kit process (Illumina). Briefly, genomic DNA was quantitatively assessed using Quant-iT picogreen dsDNA assay kit (Invitrogen, USA). The sample was normalised to the concentration of 1 ng/μL. 10 ng of DNA was used for library preparation. After tagmentation, the tagmented DNA was amplified using the facility's custom-designed i7 or i5 barcodes, with 12 cycles of PCR. The quality control for the samples was done by sequencing a pool of samples using MiSeq V2 nano kit - 300 cycles. After library amplification, 3 μL of each library was pooled into a library pool. The pool was then cleaned up using SPRI beads following the Nextera Flex clean-up and size selection protocol. The pool was then sequenced using a MiSeq V2 nano kit (Illumina, USA). Based on the sequencing data generated, the read count for each sample was used to identify the failed libraries (i.e., libraries with less than 100 reads).

Moreover, libraries were pooled at different amounts based on the read count to ensure equal representation in the final pool. The final pool was sequenced on Illumina NovaSeq 6000 Xp S4 lane, 2 × 150 bp. WGS read quality was assessed using FASTQC (version 0.11.5) and trimmed using Trimmomatic (version 0.36) with default parameters and trimmed of adaptor sequences (TruSeq3 paired-ended). Reads were aligned to the *E. coli* MG1655 genome (http://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655_gca_000005845/Info/Index/, assembly ASM584v2) and then analysed variants following GATK Best Practices for Variant Discovery (HaplotypeCaller) (64). Further genome variant annotation was conducted using the software SnpEff (65).

Global transcriptome sequencing

After ampicillin treatment for 0 and 8, surviving isolates were immediately washed and harvested for global transcriptome sequencing. Total RNA was extracted from the cell pellets using a PureLink RNA mini kit (Invitrogen) as per the manufacturer's instructions. The global transcriptome sequencing was processed and analysed by Genewiz, Jiangsu, China. Primers used in this work are listed in Table S4. RNA-Seq read quality was assessed using FASTQC and trimmed using Trimmomatic with default parameters. Reads were aligned to the *E. coli* MG1655 genome

(http://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655_gca_000005845/Info/Index/, assembly ASM584v2) and then counted using the RSubread aligner with default parameters (66). After mapping, differential expression analysis was carried out using strand-specific gene-wise quantification using the DESeq2 package (67). Further normalisation was conducted using RUVSeq and the RUV correction method, with $k = 1$ to correct for batch effects, using replicate samples to estimate the factors of unwanted variation (68). Absolute counts were transformed into standard z-scores for each gene over all treatments, that is, absolute read for a gene minus mean read count for that gene over all samples and then divided by the standard deviation for all counts over all samples. Genes with an adjusted *P* value (P_{adj}) of $\leqslant 0.05$ were considered differentially expressed. PseudoCAP analysis was conducted by calculating the percentage of genes in each classification that were differentially expressed ($\log_2 FC \geqslant \pm 2$, $P_{adj} \leqslant 0.05$).

Single-molecule localisation imaging and data analysis

Single-molecule localisation imaging was performed on a custom-built Stochastic Optical Reconstruction Microscope (STORM) with an Olympus IX81 microscope frame, a 100x magnification NA 1.45 objective (Olympus) and an EMCCD camera (DU-897, Andor) as described previously (69-71). In summary, 35 mm cell culture dishes (0.17 mm No.1 coverglass) were cleaned with 1 M KOH for 30 minutes in an ultrasonic cleaning machine, followed by three washes with MilliQ water. The dishes were air-dried with high-purity nitrogen blowing and sterilised by UV exposure for 30 minutes. *E. coli* cells were fixed with NaPO4 (30 nM), formaldehyde (2.4%), and glutaraldehyde (0.04%) at room temperature for 15 minutes, followed by 45 minutes on ice. Samples were then centrifuged to collect the pellet cells, and the supernatant was discarded. Cell pellets were washed twice with phosphate-buffered saline (PBS), pH 7.4. Cells were resuspended in 200 µL of GTE buffer and kept on ice until 200 µL was placed onto the coverslip bottom of the cleaned 35 mm culture dish. To label the bacterial chromosome, a Click-iT EdU kit was used prior to fixation following the manufacturer's instruction (ThermoFisher) and as described before. To label DNA polymerase I, fixed cells were blocked and permeabilised with blocking buffer (5% wt/vol bovine serum albumin (Sigma-Aldrich) and 0.5% vol/vol Triton X-100 in PBS) for 30 min and then incubated with 1 µg/mL primary antibody against DNA polymerase I (ab188424, Abcam) in blocking buffer for 60 min at room temperature. After washing with PBS three times, the cells were incubated with 2 µg/mL fluorescently labelled secondary antibody (Alexa 647, A20006,

ThermoFisher) against the primary antibody in the blocking buffer for 40 min at room temperature. After washing with PBS three times, the cells were postfixed with 4% (wt/vol) paraformaldehyde in PBS for 10 min and stored in PBS before imaging. STORM image analysis, drift correction, image rendering, protein cluster identification and images presentation were performed using Insight342, custom-written Matlab (2012a, MathWorks) codes, SR-Tesseler (IINS, Interdisciplinary Institute for Neuroscience) (72), and Image J (National Institutes of Health).

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.9.0.0. All data are presented as individual values and mean or mean ± SEM. A one-tailed unpaired Student's t-test using a 95% confidence interval was used to evaluate the difference between the two groups. A probability value of $P < 0.05$ was considered significant. Statistical significance is indicated in each figure. All remaining experiments were repeated independently, at least six with similar results.

Data availability

Sequence data supporting this study's findings have been deposited in the GEO repository with the GEO accession number GSE179434.

Table S1. Other mutations detected in the *ΔrecA* resistant isolates

Gene	Mutation	Genomic Position	Animo Acid Changes
stfE	A>AGGTTTTCGAGAGC	1209618	p.Val13fs
puuC	T > G	1363695	p.Phe318Cys
cpxA	T > C	4104711	p.Thr89Ala

Table S2. Strains used in this study

Strain	Relevant Genotype	Parent strain	Source
DH5α	-	-	Lab stock
E. coli MG1655	*recA*$^+$ *lexA*$^+$	-	Lab stock
LZ101	*lexA*$^+$ *ΔrecA*	MG1655	This study
LZ102	*lexA*$^+$ *ΔrecA*, pJM1071-*recA*	LZ101	This study
RW1570	*recA*$^+$ *lexA3*	MG1655	Lab stock
JW0612-1	*recA*$^+$ *lexA*$^+$ *ΔcitB*	MG1655	Keio Collection
JW0611-1	*recA*$^+$ *lexA*$^+$ *ΔcitA*	MG1655	Keio Collection
JW1134-1	*recA*$^+$ *lexA*$^+$ *Δymfm*	MG1655	Keio Collection
JW0059-1	*recA*$^+$ *lexA*$^+$ *ΔpolB*	MG1655	Keio Collection
JW0221-1	*recA*$^+$ *lexA*$^+$ *ΔdinB*	MG1655	Keio Collection
RW120	*recA*$^+$ *lexA*$^+$*ΔumuDC*	MG1655	Lab stock
EAW13	*recA*$^+$ *lexA*$^+$*ΔsulA*	MG1655	Lab stock
JW2669-1	*lexA*$^+$ *ΔrecA*	MG1655	Keio Collection

Table S3. Plasmids used in this study

Plasmid	Features	Source
pJM1071-*recA*	Low copy vector with constitutive *recA* promoter and native *recA* RBS; *recA* cloned in pJM1071 between *NdeI/XbaI*	Lab stock (59)
pKD46	Temperature sensitive replication (*repA101ts*); encodes lambda Red genes	Lab stock
pCP20	Temperature sensitive origin of replication; encodes the FLP recombinase.	Lab stock

Table S4. Primers used in this study

Name	Sequence	Function
recA-FWD	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAACAAGACGAGAAACGAGAGAGGATGCTCAC	Construction of *recA* deletion mutant
recA-REV	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAAGACGTCTAAGAAACCATTATTATCATGAC	

807

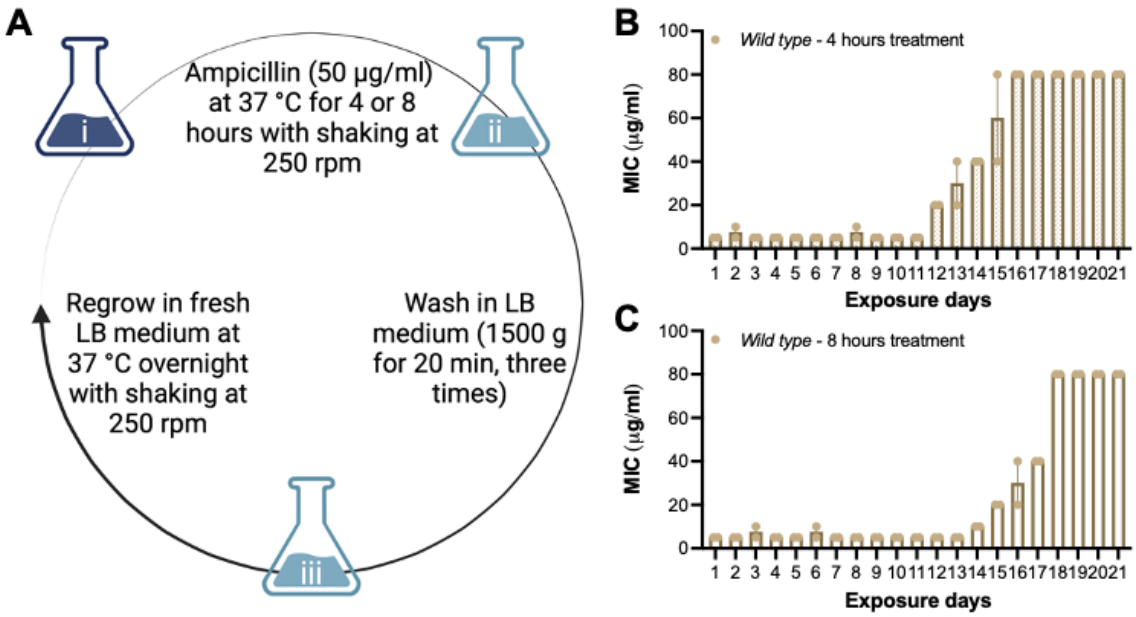


Figure S1. Long-term exposures to ampicillin induced the evolution of resistance in the wild type *E. coli* strain. (A) The experimental flow of ALE antibiotic treatment experiment. An overnight culture (0.6 mL; 1 x 10^9 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 µg/mL ampicillin and incubated at 37ºC with shaking at 250 rpm for 4 or 8 hours. After treatment, the antibiotic-containing medium was removed by washing twice (20 min centrifugation at 1500 g) in fresh LB medium. The surviving isolates were resuspended in 30 mL LB medium and grown overnight at 37ºC with shaking at 250 rpm. Ampicillin treatment was applied to the regrown culture and repeated until resistance was established. **(B)** Changes in the MICs of ampicillin in the wild type strain after 21 days of treatment with ampicillin at 50 µg/ml for 4 hours each day. (C) Changes of MICs of ampicillin in the wild type strain after 21 days of treatment with ampicillin at 50 µg/ml for 8 hours each day. MICs were measured after each daily treatment. Each experiment was independently repeated at least twice, and the data are shown as mean ± SEM.

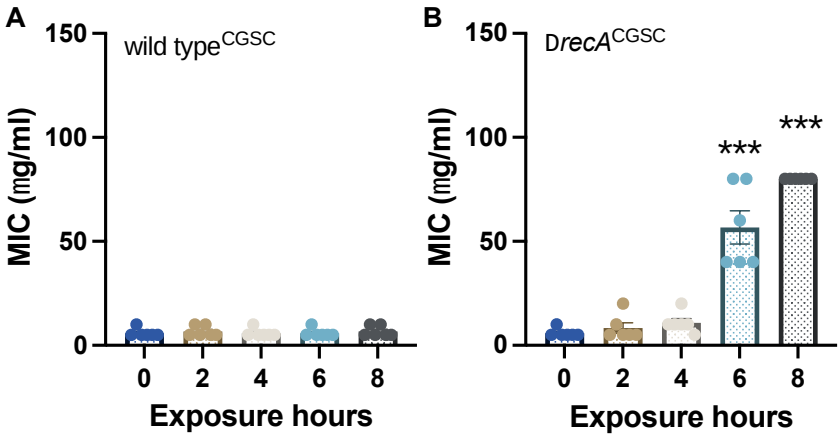


Figure S2. Single exposures to ampicillin induced the evolution of resistance in the *ΔrecA*^{CGSC} strain (JW2669-1). (A) MICs of ampicillin were measured against the wild type^{CGSC} *E coli* strain after single exposures to ampicillin. **(B)** MICs of ampicillin were measured against the *ΔrecA*^{CGSC} strain after single exposures to ampicillin. Each experiment was independently repeated at least six times using parallel replicates, and the data are shown as mean ± SEM. Significant differences among different treatment groups are analyzed by independent t-test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

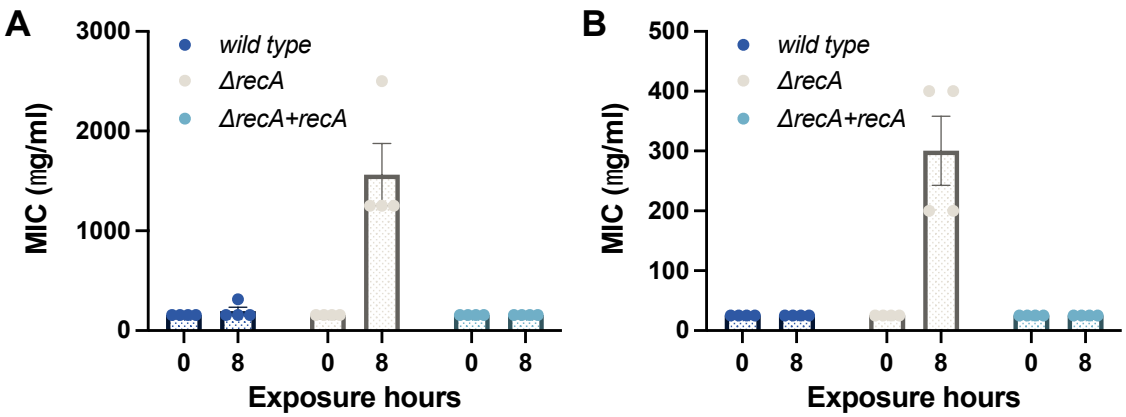


Figure S3. Single exposures to other β-lactam antibiotics induced the evolution of resistance in the *ΔrecA* strain. (A) MICs of penicillin G in the wild type, *ΔrecA*, and complemented *ΔrecA* strain, where the expression of RecA was restored, after single exposures to penicillin G at 1 mg/mL for 8 hours. **(B)** MICs of carbenicillin in the wild type, *ΔrecA*, and complemented *ΔrecA* strain, where the expression of RecA was restored, after single exposures to carbenicillin at 200 µg/mL for 8 hours. Each experiment was independently repeated at least six, and the data are shown as mean ± SEM.

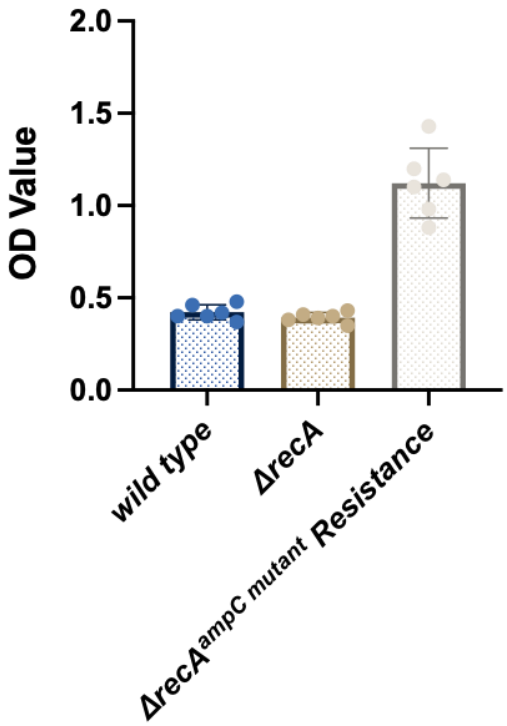


Figure S4. The activity of β-lactamase was increased in the *ΔrecA* culture supernatants. The *ΔrecA* strain was treated with ampicillin at 50 μg/ml for 8 hours, and surviving isolates harbouring the *ampC* mutations were selected. The level of β-lactamase in cell culture supernatants was determined by the absorbance at OD_{490}. The levels of β-lactamase in the wild type or *ΔrecA* strain culture supernatants without exposure to ampicillin were tested as a control. Each experiment was independently repeated six times. Each experiment was independently repeated at least six, and the data are shown as mean ± SEM.

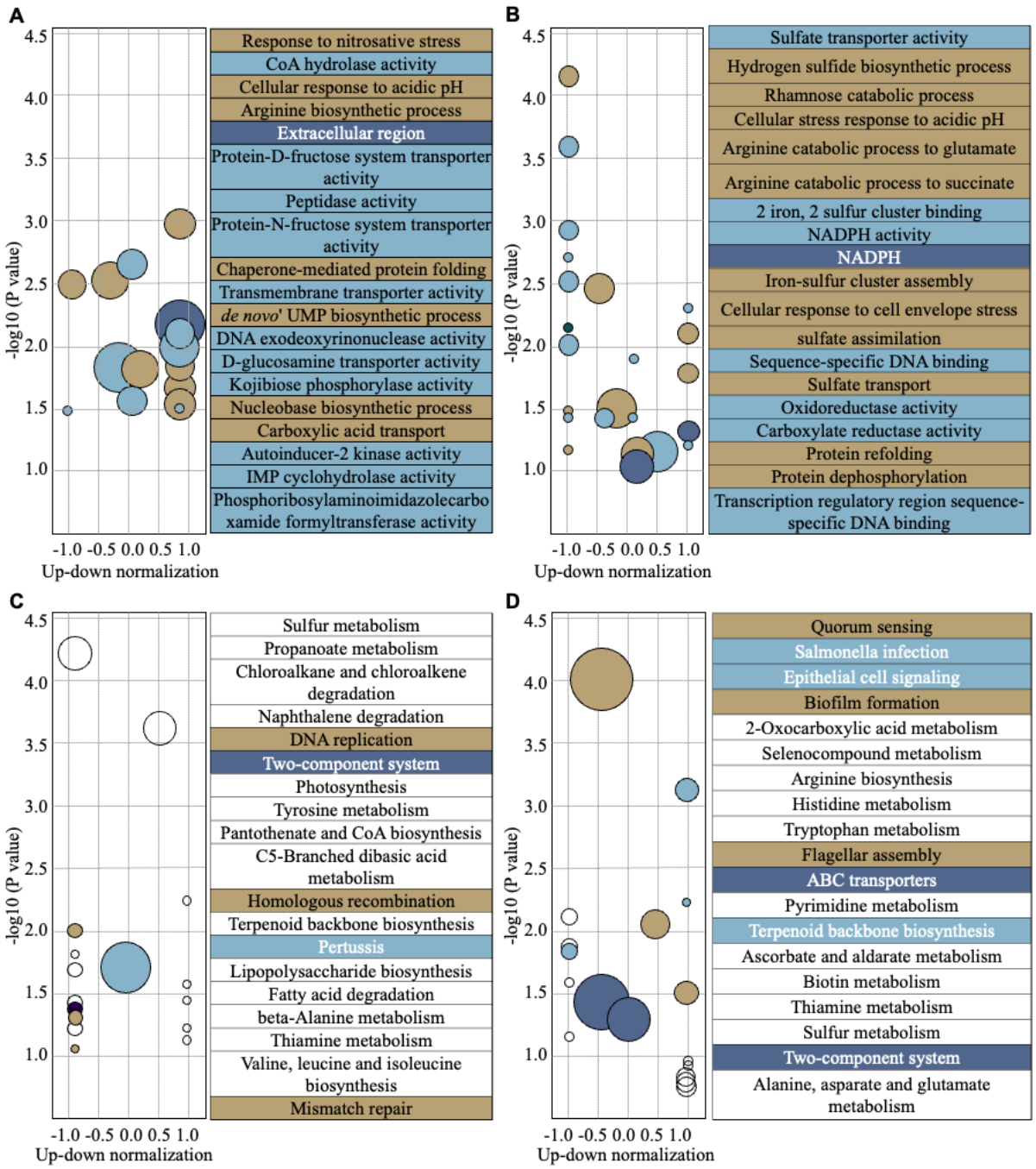


Figure S5. Transcriptional responses of the wild type and *ΔrecA* strain after single treatments with ampicillin for 8 hours. GO analysis was performed following the GOseq approach, and different genes in the wild type **(A, left)** and the *ΔrecA* strain **(B, left)** were plotted. KEGG pathway enrichment was assigned according to the KEGG database, and different genes in the wild type **(C, left)** and the *ΔrecA* strain **(D, left)** were plotted. The top 20 enrichment pathways are listed in the GO and KEGG enrichment analysis **(A-D, right)**. Total RNA-seq was performed with three repeats in each group.