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

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Abstract

The killing mechanism of many antibiotics involves the induction of DNA damage, either directly or indirectly, which activates the SOS response. RecA, the master regulator of the SOS response, has shown to play a central role in the evolution of resistance to fluoroquinolones, even after short-term exposure. While this paradigm is well established for DNA-damaging antibiotics, it remains unclear whether β-lactams elicit similar resistance dynamics or depend on RecA and SOS-mediated mechanisms. In this study, we observed a rapid and stable evolution of β-lactam resistance (20-fold MIC increase within 8 hours) in *Escherichia coli* lacking RecA after a single exposure to ampicillin. Contrary to expectation, this resistance emerged through an SOS-independent mechanism involving two distinct evolutionary forces: increased mutational supply and antibiotic-driven selection. Specifically, we found that RecA deletion impaired DNA repair and downregulated base excision repair pathways, while concurrently repressing the transcription of antioxidative defence genes. This dual impairment led to excessive accumulation of reactive oxygen species (ROS), which in turn promoted the emergence of resistance-conferring mutations. While ampicillin treatment did not alter survival, it selectively enriched for rare mutants arising in the RecA-deficient and ROS-elevated background. Collectively, our findings demonstrate that this oxidative environment, together with compromised DNA repair capacity, increases genetic instability and creates a selective landscape favouring the expansion of resistant clones. These results highlight the repair-redox axis as a key determinant of bacterial evolvability under antimicrobial stress.

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

69 has been found to contribute to the development of multidrug resistance, as prolonged exposure
70 to antibiotics in cyclic ALE experiments is known to generate ROS, leading to DNA damage
71 and increased mutagenesis (22,23). Nevertheless, there is still limited knowledge regarding the
72 consequences of ROS accumulation in bacteria when the activity of RecA or the SOS response
73 is suppressed.
74
75 Here, we report that a single exposure to β-lactam antibiotics can rapidly drive the evolution
76 of multidrug resistance in *E. coli* lacking RecA. This process reflects a two-step evolutionary
77 mechanism: RecA deficiency increases mutational supply by impairing DNA repair, repressing
78 antioxidant gene expression, and promoting ROS accumulation; subsequently, antibiotic
79 pressure selectively enriches resistant variants from this hypermutable population.
80
81 **Results**
82 **Single β-lactam exposure accelerates resistance evolution in the *recA* mutant strain**
83 **through a selection-driven mechanism**
84 To investigate the impact of the SOS response on bacterial evolution towards β-lactam
85 resistance, we generated a *recA* mutant strain (*ΔrecA*) from the *E. coli* MG1655 strain. Initially,
86 we conducted an ALE experiment using a slightly modified treatment protocol (24) on the wild
87 type and *ΔrecA* strains. During a period of three weeks, the cells were subjected to cycles of
88 ampicillin exposure for 4.5 hours at a concentration of 50 μg/mL (10 times the MIC) each day
89 (Fig. S1A) (25). As anticipated based on previous studies, the intermittent ampicillin treatments
90 over the course of three weeks resulted in the evolution of antibiotic resistance in the wild type
91 strain (Fig. S1B). However, a significantly accelerated development of resistance was found in
92 the *ΔrecA* strain, with the average time to resistance reduced to 2 days (Fig. S1B). More
93 importantly, we observed that resistance even emerged in the *ΔrecA* strain after a single
94 exposure for 8 hours to ampicillin (Fig. 1A-C). Meanwhile, after 8 hours of treatment with 50
95 μg/mL ampicillin, the survival rates of both wild type and *ΔrecA* strain were consistent (Fig.
96 S2). To ensure that the emergence of resistance we observed was not illusory due to technical
97 issues during the *recA* knockout process, we employed another *ΔrecA* strain (JW2669-1)
98 provided by the Coli Genetic Stock Centre (CGSC) with the same killing procedure. The results
99 from both bacterial strains were consistent (Fig. S3A and B). To further investigate, we treated
100 both the wild type and *ΔrecA* cells with other β-lactams, including penicillin G and carbenicillin,
101 at concentrations equivalent to 10 times the MIC (1 mg/mL and 200 μg/mL, respectively) for

102 8 hours (26,27). Consistently, these treatments also led to a fast evolution of antibiotic
103 resistance in the *ΔrecA* strain (Fig. S4A and B).
104
105 To assess the stability of this accelerated antibiotic resistance acquired by the *ΔrecA* strain, we
106 conducted a study wherein the *ΔrecA* resistant isolates, originating from the initial 8-hour
107 treatment with ampicillin, were continuously cultivated in a medium devoid of antibiotics for
108 a period of seven days. Our findings revealed that once resistance was established, resistance
109 remained stable and was able to be passed on to subsequent generations even in the absence of
110 ampicillin (Fig. 1D). Moreover, we performed a complementation experiment by introducing
111 a plasmid containing *recA* under its native promoter into the *ΔrecA* strain prior to Step i in Fig.
112 1A, that is, before exposing the cells to ampicillin. Interestingly, this complemented strain
113 displayed a comparable MIC to the isogenic wild type strain and maintained its sensitivity even
114 after ampicillin treatment for up to 8 hours (Fig. 1E).
115
116 Antibiotic resistance evolves through the combined effects of mutational events and selection
117 imposed by antimicrobial pressure (28). To clarify which mechanism underlies the rapid
118 emergence of resistance in the *ΔrecA* strain, we systematically analysed the mutation frequency
119 and distribution patterns in response to ampicillin treatment. Fig. 1F shows the distribution of
120 rifampicin-resistant colony-forming units (CFUs) across 96 independent cultures of the wild
121 type and *ΔrecA* strains, with and without exposure to ampicillin (50 μg/mL for 8 hours).
122 Although the treatment of ampicillin in the *ΔrecA* strain displayed a highly skewed distribution
123 with apparent jackpot cultures, non-parametric statistical comparison (Kruskal-Wallis with
124 Dunn's test) did not detect a significant difference in overall mutation frequency compared to
125 untreated *ΔrecA*. This suggests that while the median mutation burden remained stable, rare
126 resistant outliers were significantly enriched, which was a pattern consistent with selection
127 rather than broad mutagenesis (29). Further, using the maximum likelihood estimation (MLE)
128 (30), we calculated the mutation rates (mutations per culture) for each group (Fig. 1G). While
129 the *ΔrecA* group exhibited a modest increase in baseline mutation rate compared to the wild
130 type strain, the addition of ampicillin led to a significant increase in estimated mutation rate.
131
132 To further assess the statistical properties of these distributions, we fitted the observed data to
133 a Poisson model (Fig. S5A). The mutation frequency distributions in the wild type strain with
134 or without the treatment of ampicillin conformed well to Poisson expectations, consistent with
135 random spontaneous mutation events. In contrast, the single exposure to ampicillin

significantly deviated from the Poisson model, indicating a non-random clonal enrichment process in the *ΔrecA* strain (31). Finally, we applied a fluctuation test-based inference framework grounded in the Luria-Delbrück model to distinguish between mutation induction and clonal selection (32). As shown in Fig. S5B, only the *ΔrecA* strain treated with ampicillin exhibited a markedly non-Poisson distribution of mutant counts, characterized by a long right-skewed tail and the emergence of jackpot cultures. This distribution is inconsistent with uniform mutagenesis and instead supports a model in which antibiotic treatment selectively enriches rare early-arising resistant subpopulations. Together, these findings demonstrate that a single exposure to β-lactam antibiotics promotes a rapid and heritable evolution of antibiotic resistance in the *ΔrecA* strain, predominantly through selection rather than *de novo* induction of mutations.

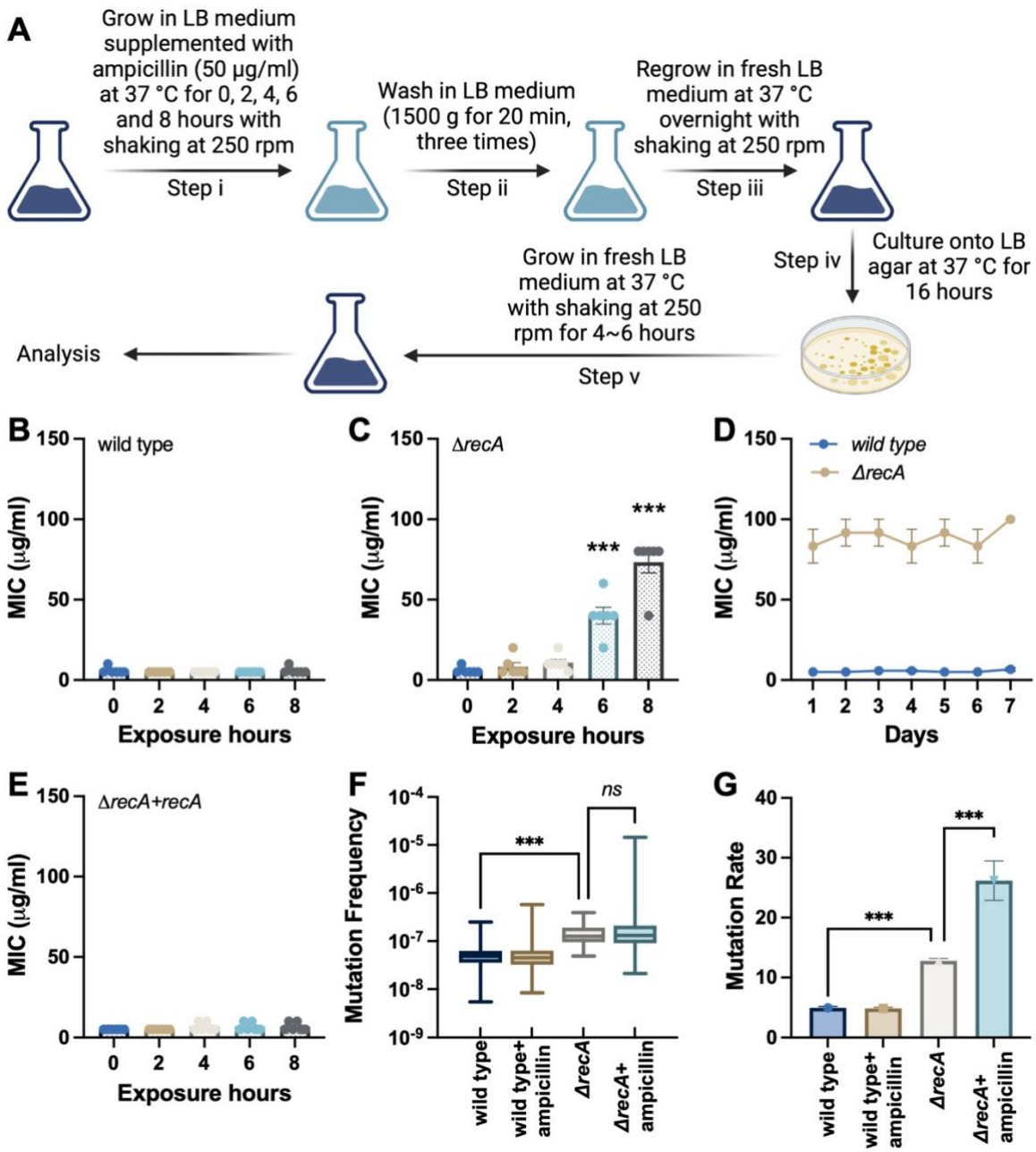
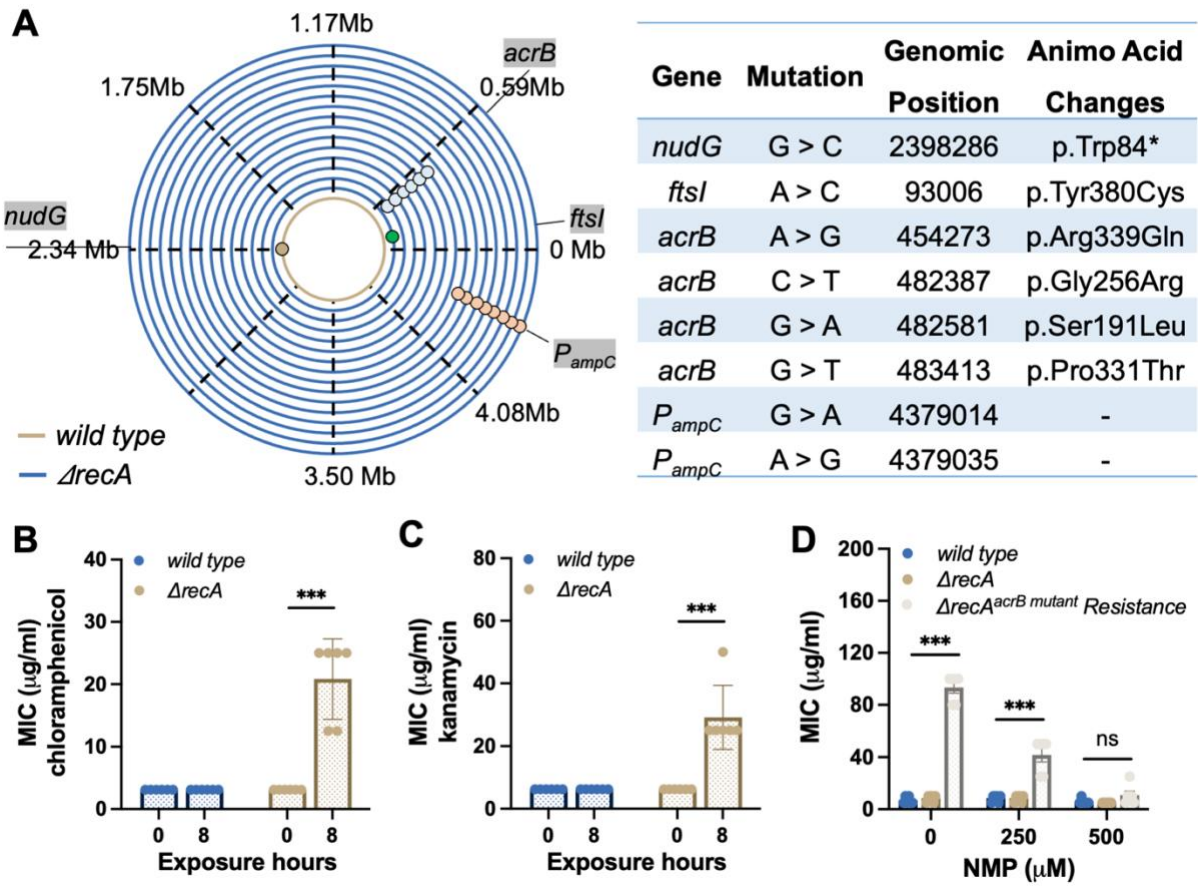


Figure 1. Fast evolution of antibiotic resistance in *E. coli recA* mutant strain. (A) Experimental flow for the single exposures to antibiotics in *E. coli* strains. Step i: an overnight culture (1 x 10^9 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 µg/mL ampicillin and incubated 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

159 ampicillin. **(C)** MICs of ampicillin were measured against the *ΔrecA* strain after single
160 exposures to ampicillin. **(D)** After the treatment of Step v, cells were continuously cultured in
161 an antibiotic-free medium for seven days. MICs of ampicillin were measured each day. **(E)**
162 MICs of ampicillin were measured against the *ΔrecA* strain treated with ampicillin, where the
163 expression of RecA was restored using plasmid-based constitutive expression of *recA* before
164 the treatment of Step i. **(F)** Distribution of rifampicin-resistant mutant counts following single
165 β-lactam exposure (96 replicate cultures in each group). Statistical comparison of mutation
166 frequency (median values) used the Kruskal-Wallis test followed by Dunn's multiple
167 comparisons. **(G)** Mutation rate (mutations per culture) estimates derived by maximum
168 likelihood analysis. Each experiment was independently repeated at least six times using
169 parallel replicates, and the data are shown as mean ± SEM. Significant differences among
170 different treatment groups are analysed by independent t-test, $*P < 0.05$, $**P < 0.01$, $***P <$
171 0.001, *ns*, no significance.
172
173 **Detection of drug resistance associated DNA mutations in *recA* mutant resistant isolates**
174 In bacteria, resistance to most antibiotics requires the accumulation of drug resistance
175 associated DNA mutations that can arise stochastically and, under stress conditions, become
176 enriched through selection over time to confer high levels of resistance (33). Having observed
177 a non-random and right-skewed distribution of mutation frequencies in *ΔrecA* isolates
178 following ampicillin exposure, we next sought to determine whether specific resistance-
179 conferring mutations were enriched in *ΔrecA* isolates following antibiotic exposure. We
180 thereby randomly selected 15 colonies on non-selected LB agar plates from the wild type
181 surviving isolates, and antibiotic screening plates containing 50 μg/mL ampicillin from the
182 *ΔrecA* resistant isolates, respectively, and performed whole-genome sequencing. We found that
183 drug resistance associated mutations were present in all resistant isolates, including the
184 mutations in the promoter of the β-lactamase *ampC* (P_{ampC}) in 8 isolates, the ampicillin-binding
185 target PBP3 (*ftsI*) in 1 isolate, and the AcrAB-TolC subunit AcrB (*acrB*) mutations in 6 isolates
186 (Fig. 2A). A mutation in gene *nudG* was detected in wild type surviving isolates after the single
187 exposure to ampicillin (Fig. 2A), which is involved in pyrimidine (d)NTP hydrolysis to avoid
188 DNA damage (34). Other mutations were listed in the Table S1.
189
190 The presence of P_{ampC} mutations was accompanied by a significant increase in the production
191 of β-lactamase in bacteria (Fig. S6). This leads to specific resistance to β-lactam antibiotics.

192 The gene *acrB* codes for a sub-component of the AcrAB-TolC multi-drug efflux pump, which
193 is central in Gram-negative bacteria (35,36). Mutations in AcrAB-TolC enhance the efflux of
194 antibiotics and confer resistance to multiple drugs (35). Consequently, after short-term
195 exposure to ampicillin, the *ΔrecA* isolates carrying the *acrB* mutations exhibited resistance to
196 other types of antibiotics, such as chloramphenicol and kanamycin (Fig. 2B and C). Treatment
197 with high concentrations of 1-(1-Naphthylmethyl) piperazine (NMP), an efflux pump inhibitor
198 (EPI) that competitively blocks TolC-composed efflux pumps, successfully restored the
199 sensitivity of *ΔrecA* resistant isolates to ampicillin, bringing them back to the equivalent
200 concentrations found in the wild type (Fig. 2D). Collectively, these results suggest that β-
201 lactam treatment rapidly selects for resistance-conferring mutations, which were enriched in
202 *ΔrecA* isolates following short-term exposure.
203

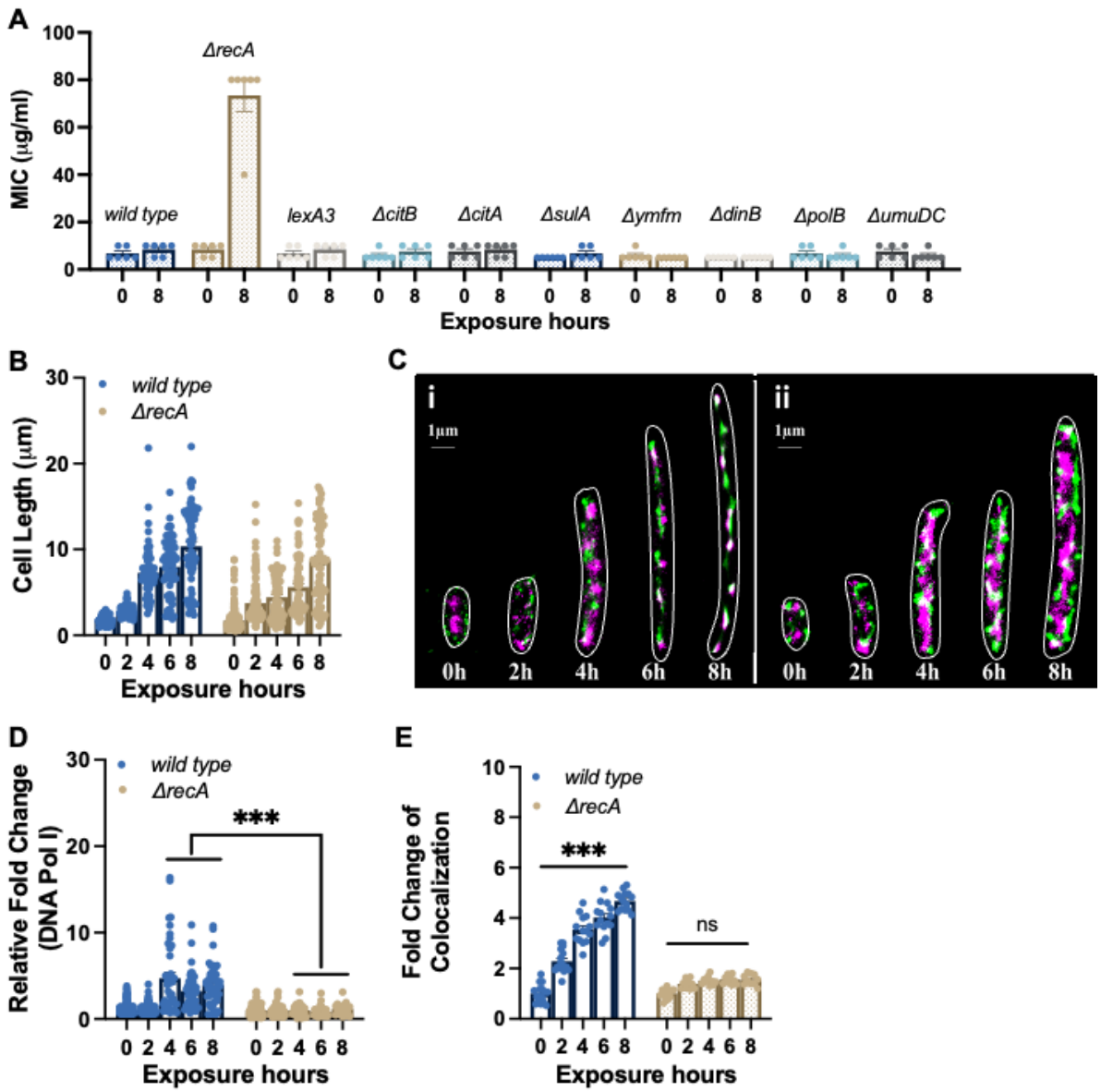


Gene	Mutation	Genomic Position	Animo Acid Changes
nudG	G > C	2398286	p.Trp84*
ftsI	A > C	93006	p.Tyr380Cys
acrB	A > G	454273	p.Arg339Gln
acrB	C > T	482387	p.Gly256Arg
acrB	G > A	482581	p.Ser191Leu
acrB	G > T	483413	p.Pro331Thr
P_{ampC}	G > A	4379014	-
P_{ampC}	A > G	4379035	-

204
205 **Figure 2. Rapid induction of drug resistance associated DNA mutations in the *recA***
206 **mutant strain. (A)** Detection of drug resistance associated DNA mutations in the wild type
207 and *ΔrecA* strain after the single exposures to ampicillin at 50 μg/mL for 8 hours. **(B)** MICs of
208 chloramphenicol against the wild type and *ΔrecA* strain after the single treatment with
209 ampicillin were tested. **(C)** MICs of kanamycin against the wild type and *ΔrecA* strain after the

210 single treatment with ampicillin were tested. **(D)** The wild type, *ΔrecA*, and *ΔrecA*$^{acrB\ mutant}$
211 resistant isolates were incubated with NMP at different concentrations for 12 hours.
212 Subsequently, MICs were tested in these strains for resistance to ampicillin. Each experiment
213 was independently repeated at least six times, and the data is shown as mean ± SEM.
214 Significant differences among different treatment groups are analyzed by independent t-test,
215 $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, *ns*, no significance.
216
217 **Hindrance of SOS-independent DNA repair in *recA* mutant resistant isolates**
218 Impairment of DNA damage repair can accelerate the accumulation of mutations and influence
219 bacterial adaptability under antibiotic stress. While RecA is best known for regulating the SOS
220 response, we asked whether its absence also impacts resistance evolution through SOS-
221 dependent or -independent repair mechanisms. To investigate it, we first tested the ability of
222 various mutants involved in different pathways of the SOS response to evolving antibiotic
223 resistance following a single treatment with ampicillin for 8 hours at 50 μg/mL. A mutant form
224 of the SOS master regulator LexA (*lexA3*), which is incapable of being cleaved and thus
225 defective in SOS induction, did not exhibit antibiotic resistance evolution (Fig. 3A).
226 Additionally, the deletion of either DpiB or DpiA (encoded by *citB* or *citA*, respectively),
227 inhibiting the DpiBA two-component signal transduction system, did not result in the
228 development of antibiotic resistance after ampicillin exposure (Fig. 3A). Moreover, the
229 deletion of several downstream effectors of the SOS response, including those involved in cell
230 division inhibition (SulA and YmfM encoded by *sulA* and *ymfM*) (37) (Fig. 3A), and DNA
231 repair (DNA Pol II, DNA Pol IV, and DNA Pol V encoded by *polB*, *dinB* and *umuDC*) (38)
232 also did not lead to the evolution of antibiotic resistance (Fig. 3A). These results indicate that
233 the rapid resistance evolution observed in the *ΔrecA* strain is not mediated by SOS pathway
234 mutants, suggesting that RecA governs resistance evolution through SOS-independent
235 mechanisms.
236
237 In addition to the DNA repair components associated with the SOS response, DNA Pol I plays
238 a role in processing RNA primers during lagging-strand synthesis and filling small gaps during
239 DNA repair reactions (39). Since DNA Pol I (encoded by *polA*) has been demonstrated as an
240 essential gene required for the growth of *E. coli* in rich medium, including the LB medium
241 (40,41), we next utilized Single Molecule Localization Microscopy (SMLM) to precisely locate
242 the chromosome and DNA Pol I in a dynamic manner. During an 8 hour exposure to ampicillin,

243 we observed the formation of multinucleated filaments in both the wild type and *ΔrecA* strain,
244 indicating a pause in cell division and suggesting a time period for bacterial DNA repair to take
245 place (Fig. 3B and C) (42). However, the expression level of DNA Pol I was significantly
246 suppressed in the *ΔrecA* strain compared to the wild type strain after 4 hours of ampicillin
247 exposure (Fig. 3D). More notably, the super-resolution colocalization analysis revealed a
248 significantly lower ratio of co-localization between the chromosome and DNA Pol I in the
249 *ΔrecA* strain (Fig. 3E). Together, these findings demonstrate that the RecA loss impairs DNA
250 repair capacity beyond SOS regulon. This repair deficiency contributes to genetic instability
251 and is able to facilitate the rapid evolution of antibiotic resistance through SOS-independent
252 pathways.
253



254

255 **Figure 3. SOS-independent impairment of DNA repair in *ΔrecA* resistant isolates. (A)**
256 MICs of ampicillin were tested against the wild type strain, *ΔrecA* strain, and mutants lacking
257 specific genes from the SOS response after single exposures to ampicillin at 50 μg/mL for 8
258 hours. **(B)** Filament cell lengths in the wild type (*n=253*) and the *ΔrecA* strain (*n=216*) after
259 single treatments with ampicillin at 50 μg/mL. **(C)** Multinucleated filaments were observed in
260 the wild type (i) and the *ΔrecA* (ii) strain after single exposures to ampicillin at 50 μg/mL.
261 Purple: *E. coli* chromosome; green: DNA Pol I. **(D)** Relative fold changes of DNA Pol I in the
262 wild type and *ΔrecA* strain after single treatments with ampicillin at 50 μg/mL. **(E)** Co-
263 localization between the *E. coli* chromosome and DNA Pol I in the wild type and *ΔrecA* strain
264 after the single exposures to ampicillin at 50 μg/mL. Data is shown as mean ± SEM. Significant
265 differences among different treatment groups are analyzed by independent t-test, $*P < 0.05$,
266 $**P < 0.01$, $***P < 0.001$, *ns*, no significance.
267
268 **Repression of antioxidative gene expression promotes the evolution of antibiotic**
269 **resistance in the *recA* mutant strain**
270 To further comprehend the fast evolution of β-lactam resistance observed in this study, we
271 investigated the gene expression changes induced by ampicillin using a comprehensive
272 transcriptome sequencing approach (total RNA-seq). Our analysis revealed significant
273 transcriptomic alterations in both the wild type and *ΔrecA* strain isolates following a single
274 treatment with ampicillin, compared to untreated controls (Fig. 4A). Specifically, we identified
275 changes in the expression of 161 and 248 coding sequences (with $\log_2 FC > 2$ and P value <
276 0.05) in the wild type and *ΔrecA* strains, respectively. Principal component analysis (PCA)
277 demonstrated a notable disparity in the effects of ampicillin on the *ΔrecA* strain compared to
278 the wild type strain (Fig. 4B). Additionally, Venn diagram analysis confirmed that 138 and 225
279 genes were uniquely regulated by ampicillin exposure in the wild type and *ΔrecA* strains,
280 respectively (Fig. 4C).
281
282 To elucidate the differential expression of genes associated with specific biological functions,
283 we conducted Gene Ontology (GO) enrichment analyses (Fig. S7A and B) and Kyoto
284 Encyclopedia of Genes and Genomes (KEGG) pathway analyses (Fig. S7C and D). Our
285 findings indicate that ampicillin profoundly impacted persistence pathways in the wild type
286 strain, specifically affecting pathways related to quorum sensing, flagellar assembly, biofilm
287 formation, and bacterial chemotaxis (43,44). Conversely, in the *ΔrecA* strain, a distinct

288 functional category associated with the oxidative stress response exhibited significant and
289 unique down-regulation. This category included activities such as sulfate transporter activity,
290 iron-sulfur cluster assembly, oxidoreductase activity, and carboxylate reductase activity.
291
292 To identify specific genes showing significant fold changes ($\log_2$FC > 2 and *P* value < 0.05),
293 we used volcano plots to visualize the comprehensive changes in gene expression across the
294 genome (Fig. 4D). We examined the transcription levels associated with the SOS response
295 system and found that the transcription of several proteins in the wild type strain can be
296 significantly induced by the single exposure to ampicillin, including *citB* and *dinB* (Fig. 4E).
297 However, in the *recA* mutant strain, antibiotic exposure does not affect the transcription levels
298 of any SOS system-related proteins, suggesting that antibiotic exposure induced the SOS
299 response in the wild type strain but not in the *ΔrecA* strain. More importantly, we discovered
300 that the induction of the transcription level of the DNA Pol I was significantly suppressed after
301 the single treatment of ampicillin in the *ΔrecA* strain compared with that in the wild type strain
302 (Fig. 4E). This is consistent with our imaging results and further supports the notion that an
303 SOS-independent evolutionary mechanism dominates the development of antibiotic resistance
304 in *recA* mutant *E. coli*.
305
306 Further, significant downregulation in the transcription of antioxidative-related genes in the
307 *ΔrecA* strain was detected, including *cysJ*, *cysI*, *cysH*, *soda*, and *sufD* (Fig. 4F). This
308 downregulation suggested an excessive accumulation of reactive oxygen species (ROS) due to
309 compromised cell antioxidative defences. It has been previously reported that the induction of
310 mutagenesis can be stimulated by the overproduction of ROS during antibiotic administration,
311 leading to the evolution of antibiotic resistance both *in vivo* and *in vitro* (22,23). Therefore, we
312 hypothesized that elevated ROS levels facilitate the rapid evolution of antibiotic resistance in
313 the *ΔrecA* strain by increasing genetic instability and enabling the selection of resistant variants.
314 To test it, we first examined the level of ROS generation in the wild type and *ΔrecA* strains
315 treated with ampicillin for 8 hours by using the fluorescent probe DCFDA/H2DCFDA. We
316 found that ROS levels significantly increased in both the wild type and *ΔrecA* strain after 8
317 hours of ampicillin treatment. However, ROS levels in the *ΔrecA* strain showed a significant
318 further increase compared to the wild type strain (Fig. 4G). Additionally, with the addition of
319 50 mM glutathione (GSH), a natural antioxidative compound, no significant change in ROS
320 levels was observed in either the wild type or *ΔrecA* strain before and after ampicillin treatment
321 (Fig. 4H). Further, we supplemented the wild type and *ΔrecA* strains with 50 mM GSH and

322 treated them with ampicillin at a concentration of 50 μg/ml for 8 hours. Remarkably, the
323 addition of GSH prevented the development of resistance to ampicillin in the *ΔrecA* strain (Fig.
324 4I), without impairing the bactericidal effectiveness of ampicillin (Fig. 4J).
325
326 Apart from the SOS response, bacterial cells coordinate other DNA repair activities through a
327 network of regulatory pathways, including base excision repair (BER) (45-47). The excessive
328 generation of ROS results in elevated levels of deoxy-8-oxo-guanosine triphosphate (8-oxo-
329 dGTP), an oxidized form of dGTP that becomes both highly toxic and mutagenic upon
330 integration into DNA. The presence of 8-oxo-dG can induce SNP mutations, especially those
331 occurring in guanine, which can be actively rectified by the BER repair pathway (47). Notably,
332 BER glycosylases MutH and MutY can identify and repair these 8-oxo-dG-dependent
333 mutations; however, when MutY and MutH are inactivated, unrepaired 8-oxo-dG can lead to
334 the accumulation of SNP mutations within cells (47). As a result, we conducted a further
335 assessment of the transcription levels of the BER repair pathway in both the wild type and
336 *ΔrecA* strain before and after a single exposure to ampicillin. We discovered that after an 8-
337 hour treatment of ampicillin, three DNA repair-associated proteins, including MutH, MutY,
338 and MutM, were notably suppressed in the *ΔrecA* strain (Fig. 4K).
339
340 Finally, we sequenced the surviving *ΔrecA* isolates and found that the addition of GSH
341 inhibited drug resistance associated mutations in the *ΔrecA* strain, which was detected in the
342 *ΔrecA* resistant isolates including genes of the promoter of *ampC* and *acrB* (Fig. 4L). Given
343 the DNA repair impairment resulting in the generation of ROS, it would have been expected
344 for genes involved in the oxidative stress response to be induced in RecA-deficient cells.
345 However, the repression of antioxidative-related genes indicated the involvement of
346 transcriptional repressors that might be regulated by RecA. Consequently, we examined the
347 transcription levels of all transcriptional repressors in both the wild type and *ΔrecA* strain.
348 Remarkably, we observed a significant upregulation of H-NS, a crucial transcriptional
349 repressor (Fig. 4M). This finding suggests that the upregulation of H-NS could potentially
350 contribute to the suppression of antioxidant gene expression in the *ΔrecA* strain, thereby
351 promoting ROS accumulation and subsequent resistance evolution. Together, these findings
352 demonstrate that RecA deficiency not only impairs DNA repair but also suppresses the
353 oxidative stress response, leading to elevated ROS and increased mutational load. This
354 oxidative-genetic imbalance forms the basis for enhanced mutational supply in RecA-deficient
355 cells

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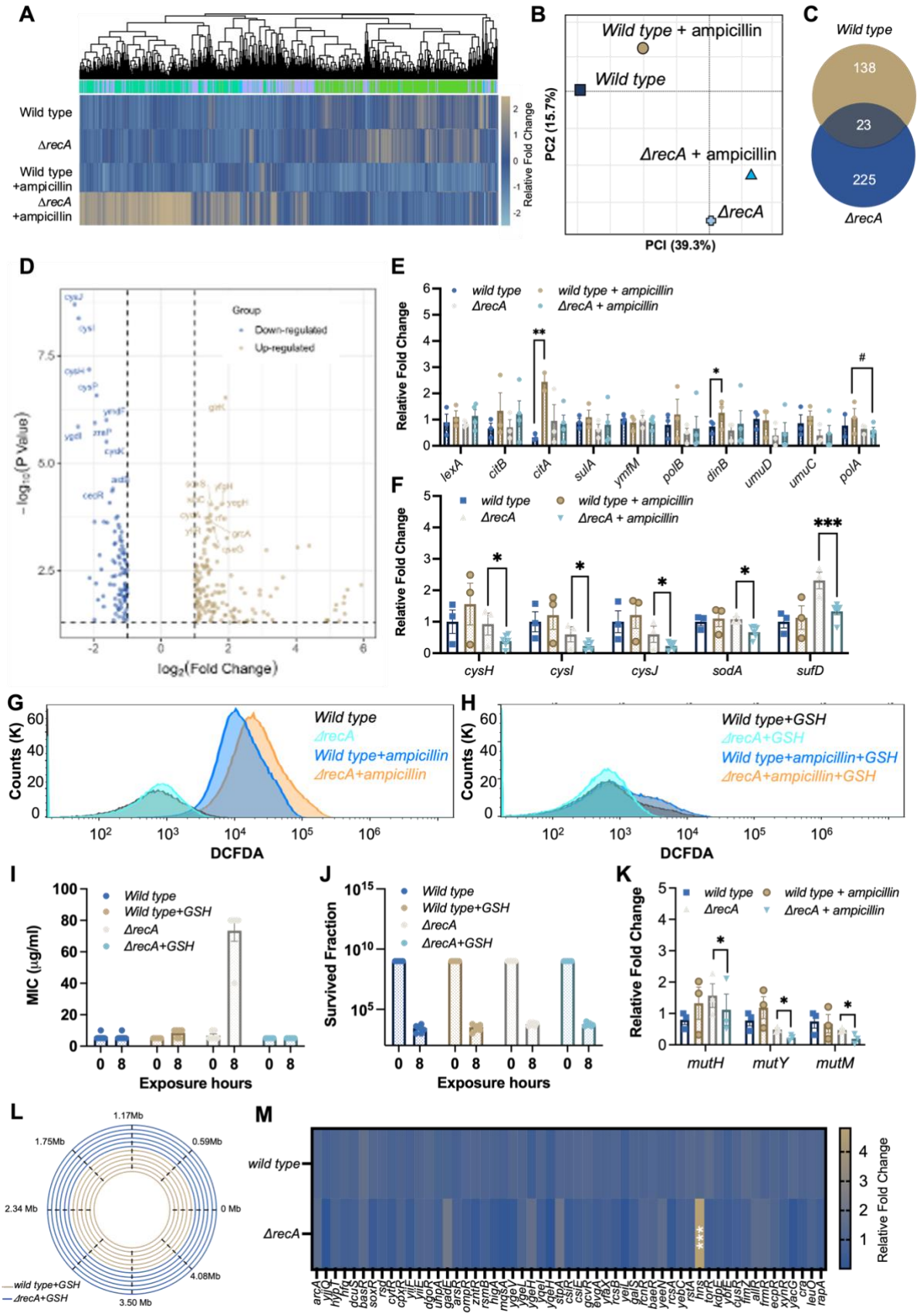
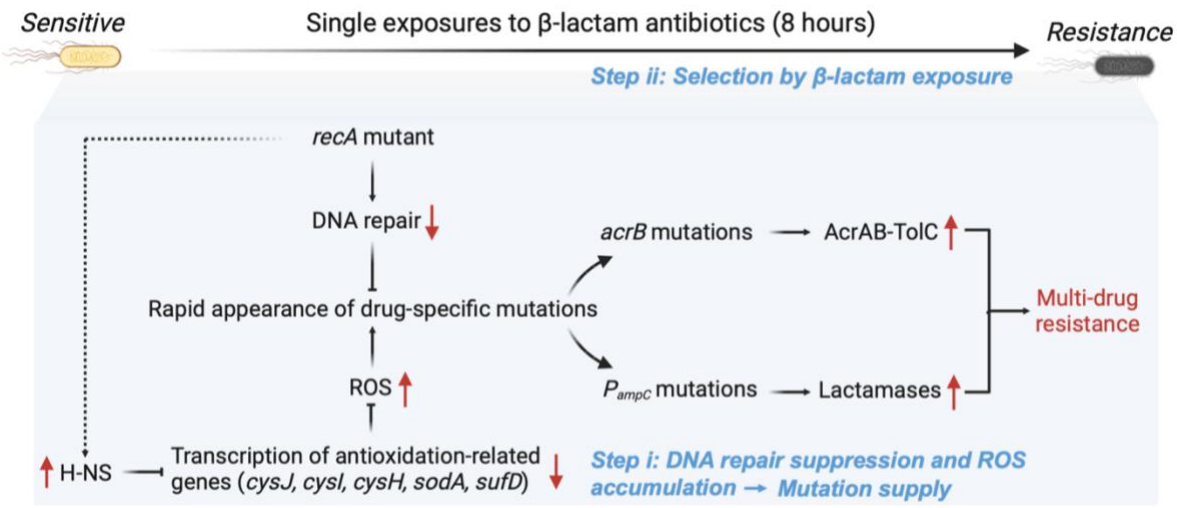

A
Wild type
ΔrecA
Wild type +ampicillin
ΔrecA +ampicillin
Relative Fold Change
B
Wild type + ampicillin
Wild type
ΔrecA + ampicillin
ΔrecA
PC2 (15.7%)
PCI (39.3%)
C
Wild type
138
23
225
ΔrecA
D
Group
Down-regulated
Up-regulated
-log10(P Value)
log2(Fold Change)
E
wild type
ΔrecA
wild type + ampicillin
ΔrecA + ampicillin
Relative Fold Change
lexA
citB
citA
sulA
ymfM
polB
dinB
umuD
umuC
polA
F
wild type
ΔrecA
wild type + ampicillin
ΔrecA + ampicillin
Relative Fold Change
cysH
cysI
cysJ
sodA
sufD
G
Counts (K)
Wild type
ΔrecA
Wild type+ampicillin
ΔrecA+ampicillin
DCFDA
H
Counts (K)
Wild type+GSH
ΔrecA+GSH
Wild type+ampicillin+GSH
ΔrecA+ampicillin+GSH
DCFDA
I
MIC (μg/ml)
Wild type
Wild type+GSH
ΔrecA
ΔrecA+GSH
Exposure hours
J
Survived Fraction
Wild type
Wild type+GSH
ΔrecA
ΔrecA+GSH
Exposure hours
K
Relative Fold Change
wild type
ΔrecA
wild type + ampicillin
ΔrecA + ampicillin
mutH
mutY
mutM
L
1.17Mb
0.59Mb
0 Mb
4.08Mb
3.50 Mb
2.34 Mb
1.75Mb
wild type+GSH
ΔrecA+GSH
M
wild type
ΔrecA
Relative Fold Change

357

358

359 **Figure 4. Overaccumulation of ROS drives the fast evolution of multi-drug resistance in**
360 **the *ΔrecA* strain. (A)** Clustered heatmap of relative expression of coding sequences in the wild
361 type and *ΔrecA* strain with significant fold changes ($\log_2$FC > 2 and *P* value < 0.05). **(B)**
362 Principal-component analysis (PCA) of normalised read counts for all strains. **(C)** Venn
363 diagram of differentially expressed genes ($\log_2$FC > 2) after treatment with ampicillin at 50
364 μg/ml for 8 hours in the wild type and *ΔrecA* strain. **(D)** The top 10 most differentially
365 expressed genes in the *ΔrecA* strain after the single treatment with ampicillin are labelled in
366 each plot. Blue dots indicate genes with a significant downregulation compared to the untreated
367 control ($\log_2$FC > 2 and *P* value < 0.05), and yellow dots indicate genes with a significant
368 upregulation compared to the untreated control ($\log_2$FC > 2 and *P* value < 0.05). **(E)** Levels of
369 transcription of SOS response system-associated genes and gene *polA* in the wild type and
370 *ΔrecA* strain after single exposures to ampicillin for 8 hours. **(F)** Levels of transcription of
371 different antioxidative associated genes in the wild type and *ΔrecA* strain after single exposures
372 to ampicillin for 8 hours. **(G)** ROS levels were measured by flow cytometry before and after 8
373 hours of ampicillin treatment (50 μg/mL) in the wild type and *ΔrecA* strains. **(H)** ROS levels
374 were measured by flow cytometry in the wild type and *ΔrecA* strains before and after 8 hours
375 of ampicillin treatment at 50 μg/mL with the addition of GSH (50 mM). **(I)** The addition of 50
376 mM antioxidative compound GSH prevented the evolution of antibiotic resistance to ampicillin
377 in the *ΔrecA* strain treated with ampicillin at 50 μg/ml for 8 hours. **(J)** Survival fraction after a
378 single exposure to ampicillin at 50 μg/ml for 8 hours in the wild type and the *ΔrecA* strain with
379 or without the addition of GSH at 50 mM. **(K)** Levels of transcription of proteins involved in
380 the BER DNA repair system in the wild type and *ΔrecA* strain after single exposures to
381 ampicillin for 8 hours. **(L)** Whole genome sequencing confirms undetectable DNA mutations
382 in the wild type and *ΔrecA* strain treated with single exposures to ampicillin with the addition
383 of GSH at 50 mM for 8 hours. **(M)** Transcription levels of all transcriptional repressors in the
384 wild type and *ΔrecA* strain after single treatments with ampicillin for 8 hours. Total RNA-seq
385 was performed with three repeats in each group. Each experiment was independently repeated
386 at least six times, and the data are shown as mean ± SEM. Significant differences among
387 different treatment groups are analyzed by independent t-test, **P* < 0.05, ***P* < 0.01, ****P* <
388 0.001, #*P* < 0.05.
389
390 **Discussion**

391 In this study, we challenge the prevailing view that disabling RecA and thereby inhibiting the
392 SOS response can prevent bacteria from developing antibiotic resistance (10). While the SOS
393 response and RecA have been extensively studied for their roles in antibiotic resistance
394 evolution (48), we observed a remarkably fast and stable evolution of multidrug resistance in
395 the *E. coli ΔrecA* strains following a single exposure to β-lactam antibiotics. This phenomenon
396 cannot be explained by canonical SOS-driven mutagenesis (49), but instead reflects the
397 interplay of two distinct evolution forces: RecA loss increases mutational supply through DNA
398 repair suppression and ROS accumulation, while antibiotic-induced lethal stress provides a
399 selective environment that promotes the expansion of rare and resistance-conferring variants
400 (Fig. 5).
401



402
403 **Figure 5. Mechanism of rapid development of multidrug resistance in the *recA* mutant *E.***
404 ***coli* strain.** Deletion of *recA* impairs DNA damage repair and upregulates the transcription of
405 the global repressor H-NS, though the mechanism of this regulation remains unclear. Elevated
406 H-NS levels repress the expression of multiple antioxidant-related genes (*cysI*, *cysJ*, *cysH*,
407 *sodA*, *sufD*), leading to excessive accumulation of ROS. The resulting oxidative stress increases
408 the overall mutational burden. Upon single exposure to β-lactam antibiotics, drug-resistant
409 subpopulations carrying specific mutations, such as in *acrB* or P_{ampC}, are selectively enriched,
410 ultimately driving the rapid emergence of multidrug resistance. This process reflects a two-step
411 mechanism involving enhanced mutational supply and subsequent selection under antibiotic
412 pressure.
413
414 To determine whether antibiotic exposure in *ΔrecA* cells directly induces mutations or
415 selectively enriches resistant variants, we combined statistical modelling, fluctuation analysis,

416 and whole-genome sequencing. Although mutation rates estimated by maximum likelihood
417 were moderately elevated in *ΔrecA* cells after ampicillin treatment, the mutation frequency
418 distributions were highly right-skewed and deviated from Poisson expectations, a hallmark of
419 clonal selection rather than uniform and population-wide mutagenesis. These findings align
420 with the classical Luria-Delbrück framework and indicate that resistance evolution in *ΔrecA* is
421 primarily driven by selection (29). However, our data also demonstrate that the mutational
422 supply is enhanced in this background due to antibiotic-induced oxidative stress and impaired
423 DNA repair, which together increase the likelihood that resistance-conferring mutations arise
424 and persist. Thus, this process reflects a selection-dominated evolutionary trajectory facilitated
425 by stress-enhanced mutagenesis, rather than classical induced mutation or selection on pre-
426 existing variants.
427
428 Mechanistically, our data reveal that RecA plays broader roles in genome stability beyond its
429 function in SOS activation. Disruption of individual SOS components, including *lexA*, *citA/B*,
430 or translesion polymerases, did not recapitulate the *ΔrecA*-specific resistance phenotype.
431 Instead, *ΔrecA* strains exhibited suppression of DNA Pol I. and key BER genes, such as *mutH*,
432 *mutY*, and *mutM*, confirming that RecA is essential for the maintenance of repair fidelity under
433 stress conditions. This defect likely enables ROS-induced DNA lesions to persist and become
434 fixed as mutations.
435
436 In parallel, we identified a striking repression of antioxidative stress response genes in *ΔrecA*
437 strains exposed to ampicillin, including *cysJIH*, *sodA*, and *sufD*. This transcriptional
438 suppression was associated with markedly elevated ROS levels. Crucially, supplementation
439 with the antioxidant GSH reversed both ROS accumulation and the emergence of resistance,
440 without impairing the bactericidal activity of ampicillin. This decouples the mutagenic and
441 lethal effects of ROS, and highlights ROS as a driver of mutational supply rather than survival.
442
443 To explore the transcriptional basis for oxidative dysregulation, we examined global repressors
444 and discovered that *hns* was significantly upregulated in *ΔrecA* strains after ampicillin exposure.
445 H-NS is a known transcriptional silencer of stress response genes, including those involved in
446 redox regulation (50,51). We propose that RecA deficiency may lead to H-NS derepression,
447 thereby silencing antioxidant defences, exacerbating ROS accumulation, and enabling
448 mutation accumulation under stress. Although previous findings report that H-NS can down-
449 regulate the transcriptional expression of *cysIJH* through the mediation of *cysB* (52,53), the

450 mechanistic relationship between RecA and H-NS regulation remains to be experimentally
451 validated.
452
453 More broadly, our findings highlight the repair-redox axis as a central regulator of bacterial
454 evolvability. Rather than solely targeting bacterial growth or survival, future antimicrobial
455 strategies might focus on constraining mutational potential. For example, co-administration of
456 antioxidants or repair stabilizers could buffer stress-induced mutagenesis without
457 compromising antibiotic efficacy, which has been a concept already under exploration in
458 cancer therapeutics (54,55).
459
460 Clinically, the emergence of *acrB* mutations and enhanced activity of the AcrAB-TolC efflux
461 pump in *ΔrecA* strains recapitulates known multidrug resistance pathways. These observations
462 raise concerns for therapies combining DNA repair inhibitors with ROS-inducing antibiotics
463 or anticancer drugs (56). Our data suggest that such combinations may inadvertently promote
464 resistance evolution, particularly in immunocompromised patients or during chemotherapy.
465
466 Finally, ROS-driven mutagenesis and repair suppression are not unique to β-lactams.
467 Antimicrobial technologies such as antimicrobial photodynamic therapy (aPDT) and cold
468 atmospheric plasma (CAP) (57,58) also rely on oxidative stress. In RecA-deficient or stress-
469 sensitized bacteria, these approaches may risk accelerating resistance evolution unless
470 accompanied by safeguards that preserve genomic integrity. Thus, maintaining the
471 transcription of genes involved in the oxidative stress defence or combining antibiotics
472 represents a promising strategy to prevent ROS-driven mutagenesis and thereby limit the
473 evolutionary emergence of resistance during antimicrobial therapy.
474
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480
481 **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.

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668

669 **Materials and Methods**

670 **Bacterial strains, medium and antibiotics**

671 Bacterial strains and plasmids used in this work are described in Table S2 and Table S3. Luria-
672 Bertani (LB) was used as broth or in agar plates. *E. coli* cells were grown in LB liquid medium
673 or on LB agar (1.5% w/v) plates at 37ºC, unless stated otherwise, antibiotics were
674 supplemented, where appropriate. Antibiotic stock solutions were prepared by dissolving
675 antibiotics in MilliQ filter sterilising, including ampicillin (50 mg/mL), penicillin G (100
676 mg/mL), carbenicillin (20 mg/mL), kanamycin (50 mg/mL) and tetracycline (10 mg/mL).
677 Chloramphenicol stock solution was prepared in 95% EtOH (25 mg/mL). Antibiotic solutions
678 were stored at -20ºC (long-term) or 4ºC (short-term).
679

680 **Treatment with antibiotics to induce evolutionary resistance**

681 For the single exposure to antibiotic experiment, an overnight culture (0.6 mL; 1 x 10^9 CFU/mL
682 cells) was diluted 1:50 into 30 mL LB medium supplemented with antibiotics (50 µg/mL
683 ampicillin, 1 mg/mL penicillin G, or 200 µg/mL carbenicillin) and incubated at 37ºC with
684 shaking at 250 rpm for 0, 2, 4, 6 and 8 hours, respectively. After each treatment, the antibiotic-
685 containing medium was removed by washing twice (20 min centrifugation at 1500 g) in fresh
686 LB medium (See Fig. 1A for method overview).
687
688 To test resistance, the surviving isolates were first resuspended in 30 mL LB medium and
689 grown overnight at 37ºC with shaking at 250 rpm. The regrown culture was then plated onto
690 LB agar and incubated overnight at 37ºC. Single colonies were isolated and grown in LB
691 medium for 4-6 hours at 37ºC with shaking at 250 rpm, which were then used to test the
692 resistance or stored at -80ºC for future use.
693
694 For the ALE antibiotic treatment experiments, an overnight culture (0.6 mL; 1 x 10^9 CFU/mL
695 cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 µg/mL ampicillin and
696 incubated at 37ºC with shaking at 250 rpm for 4.5 hours. After treatment, the antibiotic-
697 containing medium was removed by washing twice (20 min centrifugation at 1500 g) in a fresh
698 LB medium. The remaining pellet was resuspended in 30 mL LB medium and grown overnight
699 at 37ºC with shaking at 250 rpm. Ampicillin treatment was applied to the regrown culture and
700 repeated until resistance was established, as confirmed by MIC measurement.
701
702 **Antibiotic susceptibility testing**
703 The susceptibility of *E. coli* cells to antibiotics was measured using minimum inhibitory
704 concentration (MIC) testing (60). In brief, overnight cultures were diluted and incubated at
705 37ºC for 4-6 hours with shanking at 250 rpm. Cells were then diluted 1:100 and incubated with
706 increasing concentrations of antibiotics in the Synergy HT BioTek plate reader (BioTek
707 Instruments Inc., USA) at 37ºC for 16 hours. It was programmed to measure the OD hourly at
708 595 nm (Gen5 software, BioTek Instruments Inc., USA). The minimum inhibitory
709 concentration was determined as the concentration of antibiotic where no visible growth was
710 observed.
711
712 **Survival assays**
713 Overnight cultures of *E. coli* were prepared from single colonies in LB medium and incubated
714 at 37°C with shaking (250 rpm). The overnight cultures were diluted 1:50 in fresh LB medium

715 containing 50 μg/mL ampicillin to initiate antibiotic treatment. Cultures were incubated at
716 37°C for the indicated times under shaking conditions. Following treatment, cells were
717 collected by centrifugation (1500 g, 20 minutes), and the antibiotic-containing medium was
718 removed by washing twice with fresh LB medium. Serial dilutions of the washed cultures were
719 prepared, and 25 μL of each dilution was plated onto LB agar plates. Plates were incubated
720 overnight at 37°C, and CFUs were counted the following day to evaluate bacterial survival
721 rates.
722
723 **Mutation frequency and fluctuation analysis**
724 Overnight cultures inoculated from single colonies in LB medium were diluted 1:1,000,000
725 and incubated at 37°C with shaking until the OD_{600} reached to 1~1.3. This extreme dilution
726 minimizes the presence of pre-existing stationary phase mutants and allows *de novo* mutation
727 events to occur during exponential growth. For each biological condition, 96 independent
728 parallel cultures were prepared to perform a fluctuation analysis. The total number of colony-
729 forming units per ml (CFU/ml) was determined by plating on LB agar. To identify rifampicin-
730 resistant mutants, the remaining culture volume was centrifuged and plated on LB agar
731 containing rifampicin (100 μg/mL). LB plates were incubated for 24 hours at 37°C and
732 selective plates were incubated for 48-72 hours at 37°C (61).
733
734 The mutation frequency was calculated as the ratio of CFU/ml on rifampicin-containing plates
735 to CFU/ml on non-selective LB plates for each culture. Distributions of mutation frequencies
736 across replicate cultures were plotted, and deviations from Poisson expectations were assessed
737 by model fitting.
738
739 To estimate the mutation rate (mutations per culture), maximum likelihood estimation (MLE)
740 was applied using the Ma-Sandri-Sarkar algorithm implemented in the FALCOR toolset
741 (https://www.keshavsingh.org/protocols/FALCOR.html). The inferred mutation rate
742 distributions were compared across treatment conditions, and non-Poisson distributions
743 indicative of jackpot cultures were further analysed using fluctuation test-based inference
744 following the Luria–Delbrück framework (29).
745
746 **Construction of *recA* deletion mutant**
747 Lambda Red recombination was used to generate the gene *recA* deletion in the *E. coli* K-12
748 strain, followed by previously reported methods with modifications (62,63). Primers (*recA*-

749 *FWD* and *recA-REV,* Table S4) were designed approximately 50 bp upstream and downstream
750 to the gene *recA* on the chromosome to amplify the tetracycline cassette as well as the flanking
751 DNA sequence needed for homologous recombination. Phusion polymerase (NEB) was used
752 to amplify the DNA sequence (Table S4), and the reaction was cleaned up using a PureLink™
753 PCR purification kit (ThermoFisher Scientific) as per the manufacturer's instructions. Electro-
754 competent *E. coli* MG1655 containing the recombinase plasmid pKD46 was transformed with
755 50 ng of amplified DNA a 30°C. The transformation was plated onto LB agar plates containing
756 10 μg/mL tetracycline and incubated overnight at 37°C. PCR was used to confirm the insertion
757 of the tetracycline resistance cassette at the correct site on the chromosome using primers
758 upstream and downstream to the gene *recA*. The newly constructed mutant strains were cured
759 of plasmid pKD46 by incubating LB streak plates at 42°C overnight. Loss of the plasmid was
760 confirmed by lack of ampicillin sensitivity on LB agar plates. Mutant strains were made
761 electro-competent, and 50 μL of cells were transformed with plasmid pCP20 and incubated on
762 100 μg/mL ampicillin plates at 30°C overnight. A few colonies were then restreaked onto LB
763 plates and incubated overnight at 42°C. PCR products confirmed the loss of cassette and
764 plasmid.
765
766 **β-lactamase assay**
767 The amount of β-lactamase was measured using a β-lactamase Activity Assay Kit (Sigma-
768 Aldrich, US). Briefly, cells were collected by centrifugation at 10,000 g for 10 min, and the
769 pellet was resuspended with 5 μL of assay buffer per mg of sample. Then, 48 μL of the sample
770 was mixed with 2 μL of nitrocefin. The β-Lactamase activity was monitored by measuring the
771 absorbance at 490 nm for 30 min at 28°C. The level of β-lactamase was determined by the
772 absorbance at OD_{390}.
773
774 **Whole genome sequencing**
775 Resistant clones were isolated by selection using LB agar plates with the supplementation of
776 ampicillin at 50 μg/mL, and non-resistant clones were isolated from the LB agar plates without
777 the supplementation of antibiotics. Chromosomal DNA was extracted and purified using the
778 PureLink™ Genomic DNA mini kit following the manufacturer's instructions (ThermoFisher
779 Scientific). Whole genome sequencing (WGS) was conducted following the Nextera Flex
780 library preparation kit process (Illumina). Briefly, genomic DNA was quantitatively assessed
781 using Quant-iT picogreen dsDNA assay kit (Invitrogen, USA). The sample was normalised to
782 the concentration of 1 ng/μL. 10 ng of DNA was used for library preparation. After

783 tagmentation, the tagmented DNA was amplified using the facility's custom-designed i7 or i5
784 barcodes, with 12 cycles of PCR. The quality control for the samples was done by sequencing
785 a pool of samples using MiSeq V2 nano kit - 300 cycles. After library amplification, 3 μL of
786 each library was pooled into a library pool. The pool was then cleaned up using SPRI beads
787 following the Nextera Flex clean-up and size selection protocol. The pool was then sequenced
788 using a MiSeq V2 nano kit (Illumina, USA). Based on the sequencing data generated, the read
789 count for each sample was used to identify the failed libraries (i.e., libraries with less than 100
790 reads).
791
792 Moreover, libraries were pooled at different amounts based on the read count to ensure equal
793 representation in the final pool. The final pool was sequenced on Illumina NovaSeq 6000 Xp
794 S4 lane, 2 × 150 bp. WGS read quality was assessed using FASTQC (version 0.11.5) and
795 trimmed using Trimmomatic (version 0.36) with default parameters and trimmed of adaptor
796 sequences (TruSeq3 paired-ended). Reads were aligned to the *E. coli* MG1655 genome
797 (http://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655_gca_000005845/Info
798 /Index/, assembly ASM584v2) and then analysed variants following GATK Best Practices for
799 Variant Discovery (HaplotypeCaller) (64). Further genome variant annotation was conducted
800 using the software SnpEff (65).
801
802 **Global transcriptome sequencing**
803 After ampicillin treatment for 0 and 8, surviving isolates were immediately washed and
804 harvested for global transcriptome sequencing. Total RNA was extracted from the cell pellets
805 using a PureLink RNA mini kit (Invitrogen) as per the manufacturer's instructions. The global
806 transcriptome sequencing was processed and analysed by Genewiz, Jiangsu, China. Primers
807 used in this work are listed in Table S4. RNA-Seq read quality was assessed using FASTQC
808 and trimmed using Trimmomatic with default parameters. Reads were aligned to the *E. coli*
809 MG1655 genome
810 (http://bacteria.ensembl.org/Escherichia_coli_str_k_12_substr_mg1655_gca_000005845/Info
811 /Index/, assembly ASM584v2) and then counted using the RSubread aligner with default
812 parameters (66). After mapping, differential expression analysis was carried out using strand-
813 specific gene-wise quantification using the DESeq2 package (67). Further normalisation was
814 conducted using RUVSeq and the RUV correction method, with $k = 1$ to correct for batch
815 effects, using replicate samples to estimate the factors of unwanted variation (68). Absolute
816 counts were transformed into standard z-scores for each gene over all treatments, that is,

817 absolute read for a gene minus mean read count for that gene over all samples and then divided
818 by the standard deviation for all counts over all samples. Genes with an adjusted *P* value
819 (P_{adj}) of $\leqslant 0.05$ were considered differentially expressed. PseudoCAP analysis was conducted
820 by calculating the percentage of genes in each classification that were differentially expressed
821 ($\log_2 FC \geqslant \pm 2$, $P_{adj} \leqslant 0.05$).
822
823 **ROS measurement**
824 Intracellular ROS accumulation was measured using the Cellular ROS Assay Kit (Abcam,
825 ab113851) according to the manufacturer's protocol. Overnight cultures of both wild type and
826 Δ*recA* strains were grown in LB medium. Cells were treated with 50 μg/mL ampicillin for 8
827 hours at 37°C with shaking (250 rpm). After the treatment, cells were washed twice with 1x
828 buffer to remove residual antibiotics and debris. The collected cells were resuspended in 1x
829 buffer and incubated with the fluorescent ROS probe DCFDA (2',7'–dichlorofluorescin
830 diacetate)/H2DCFDA at a final concentration of 10 μM for 30 minutes at 37°C in the dark to
831 prevent probe degradation.
832
833 After incubation, 500 μL of the stained cells were immediately analysed using a CytoFLEX
834 LX flow cytometer (Beckman Coulter). Fluorescence was detected in the FITC channel
835 (excitation at 488 nm, emission at 525 nm). Data were analysed using FlowJo software
836 (Version X, Tree Star Inc.), with fluorescence intensity serving as an indicator of intracellular
837 ROS levels. Appropriate controls, including unstained cells and cells without ampicillin
838 treatment, were included to ensure accurate ROS measurement.
839
840 **Single-molecule localisation imaging and data analysis**
841 Single-molecule localisation imaging was performed on a custom-built Stochastic Optical
842 Reconstruction Microscope (STORM) with an Olympus IX81 microscope frame, a 100x
843 magnification NA 1.45 objective (Olympus) and an EMCCD camera (DU-897, Andor) as
844 described previously (69-71). In summary, 35 mm cell culture dishes (0.17 mm No.1
845 coverglass) were cleaned with 1 M KOH for 30 minutes in an ultrasonic cleaning machine,
846 followed by three washes with MilliQ water. The dishes were air-dried with high-purity
847 nitrogen blowing and sterilised by UV exposure for 30 minutes. *E. coli* cells were fixed with
848 NaPO4 (30 nM), formaldehyde (2.4%), and glutaraldehyde (0.04%) at room temperature for
849 15 minutes, followed by 45 minutes on ice. Samples were then centrifuged to collect the pellet

850 cells, and the supernatant was discarded. Cell pellets were washed twice with phosphate-
851 buffered saline (PBS), pH 7.4. Cells were resuspended in 200 μL of GTE buffer and kept on
852 ice until 200 μL was placed onto the coverslip bottom of the cleaned 35 mm culture dish. To
853 label the bacterial chromosome, a Click-iT EdU kit was used prior to fixation following the
854 manufacturer's instruction (ThermoFisher) and as described before. To label DNA polymerase
855 I, fixed cells were blocked and permeabilised with blocking buffer (5% wt/vol bovine serum
856 albumin (Sigma-Aldrich) and 0.5% vol/vol Triton X-100 in PBS) for 30 min and then incubated
857 with 1 μg/mL primary antibody against DNA polymerase I (ab188424, Abcam) in blocking
858 buffer for 60 min at room temperature. After washing with PBS three times, the cells were
859 incubated with 2 μg/mL fluorescently labelled secondary antibody (Alexa 647, A20006,
860 ThermoFisher) against the primary antibody in the blocking buffer for 40 min at room
861 temperature. After washing with PBS three times, the cells were postfixed with 4% (wt/vol)
862 paraformaldehyde in PBS for 10 min and stored in PBS before imaging. STORM image
863 analysis, drift correction, image rendering, protein cluster identification and images
864 presentation were performed using Insight342, custom-written Matlab (2012a, MathWorks)
865 codes, SR-Tesseler (IINS, Interdisciplinary Institute for Neuroscience) (72), and Image J
866 (National Institutes of Health).
867
868 **Statistical analysis**
869 Statistical analysis was performed using GraphPad Prism v.9.0.0. All data are presented as
870 individual values and mean or mean ± SEM. Unless otherwise specified, statistical
871 comparisons were conducted using one-way ANOVA (for multiple groups) or unpaired two-
872 tailed Student's t-tests (for two-group comparisons), assuming a 95% confidence interval.. A
873 probability value of $P < 0.05$ was considered significant. Statistical significance is indicated in
874 each figure. All remaining experiments were repeated independently, at least six with similar
875 results.
876
877 **Data availability**
878 Sequence data supporting this study's findings have been deposited in the GEO repository with
879 the GEO accession number GSE179434.
880

881 **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

882

883 **Table S2. Strains used in this study**

Strain	Relevant Genotype	Parent strain	Source
DH5α	-	-	Lab stock
E. coli MG1655	*recA*$^+$ *lexA*$^+$	-	Lab stock
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

884

885 **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

886

887 **Table S4. Primers used in this study**

Name	Sequence	Function

recA-FWD	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAACAAGACGAGAAACGAGAGAGGATGCTCAC	Construction of *recA* deletion mutant
recA-REV	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAAGACGTCTAAGAAACCATTATTATCATGAC	

888

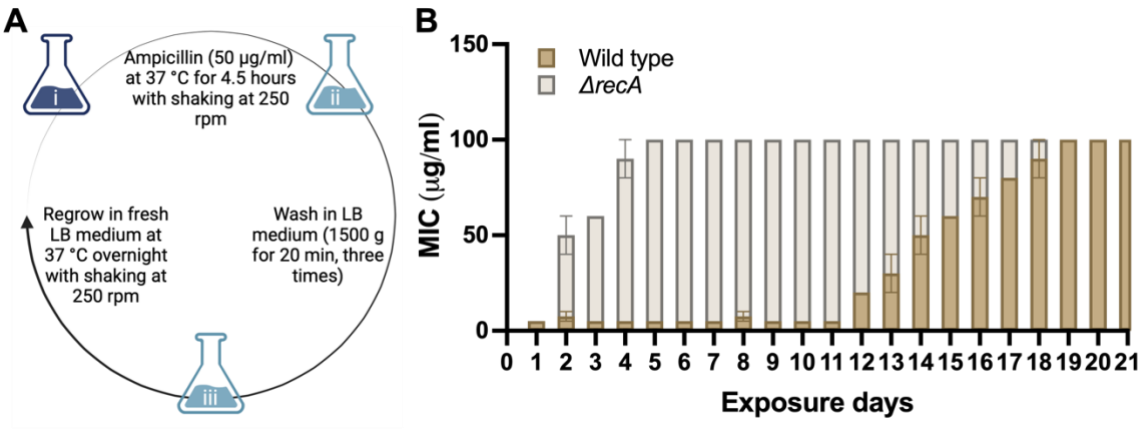


Figure S1. Long-term exposures to ampicillin induced the evolution of resistance in the wild type and *ΔrecA E. coli* strain. (A) The experimental flow of ALE antibiotic treatment experiment. An overnight culture (0.6 mL; 1 x 10^9 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 µg/mL ampicillin and incubated at 37ºC with shaking at 250 rpm for 4.5 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 and *ΔrecA* strain after 21 days of treatment with ampicillin at 50 µg/ml for 4.5 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.

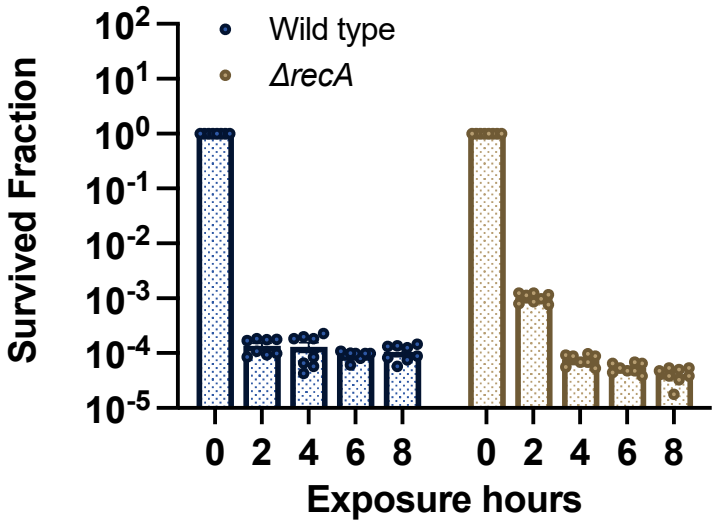


Figure S2. The survival rate after a single exposure to ampicillin at 50 µg/ml for 0,2,4,6, and 8 hours in the wild type and *ΔrecA* strain.

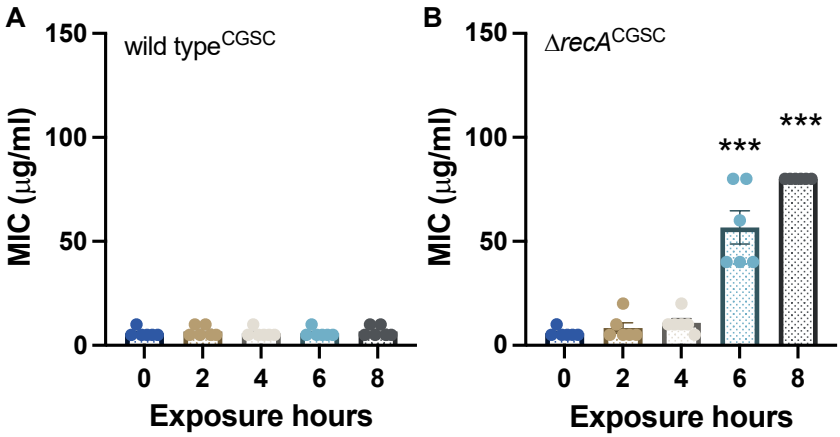


Figure S3. Single exposures to ampicillin induced the evolution of resistance in the *ΔrecA*^{CGSC} strain (JW2669-1). (A) MICs of ampicillin were measured against the wild type^{CGSC} *E coli* strain after single exposures to ampicillin. **(B)** MICs of ampicillin were measured against the *ΔrecA*^{CGSC} 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, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

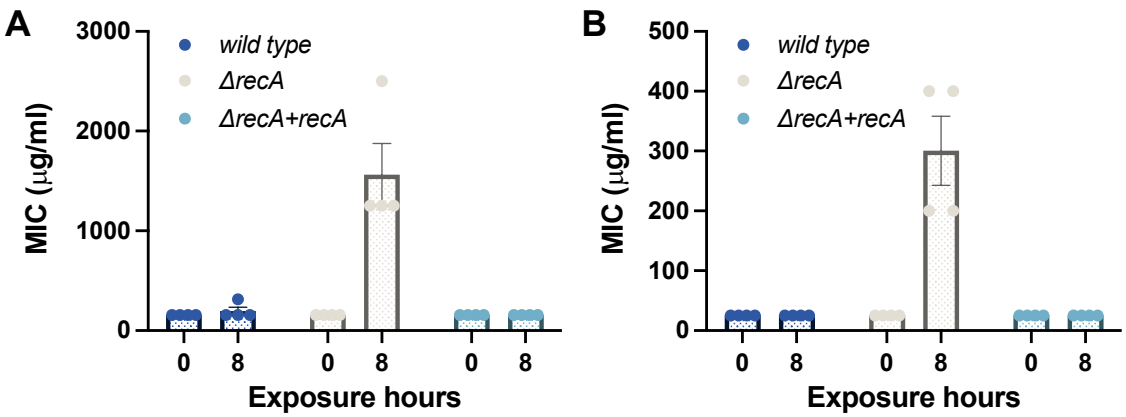


Figure S4. Single exposures to other β-lactam antibiotics induced the evolution of resistance in the *ΔrecA* strain. (A) MICs of penicillin G in the wild type, *ΔrecA*, and complemented *ΔrecA* strain, where the expression of RecA was restored, after single exposures to penicillin G at 1 mg/mL for 8 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.

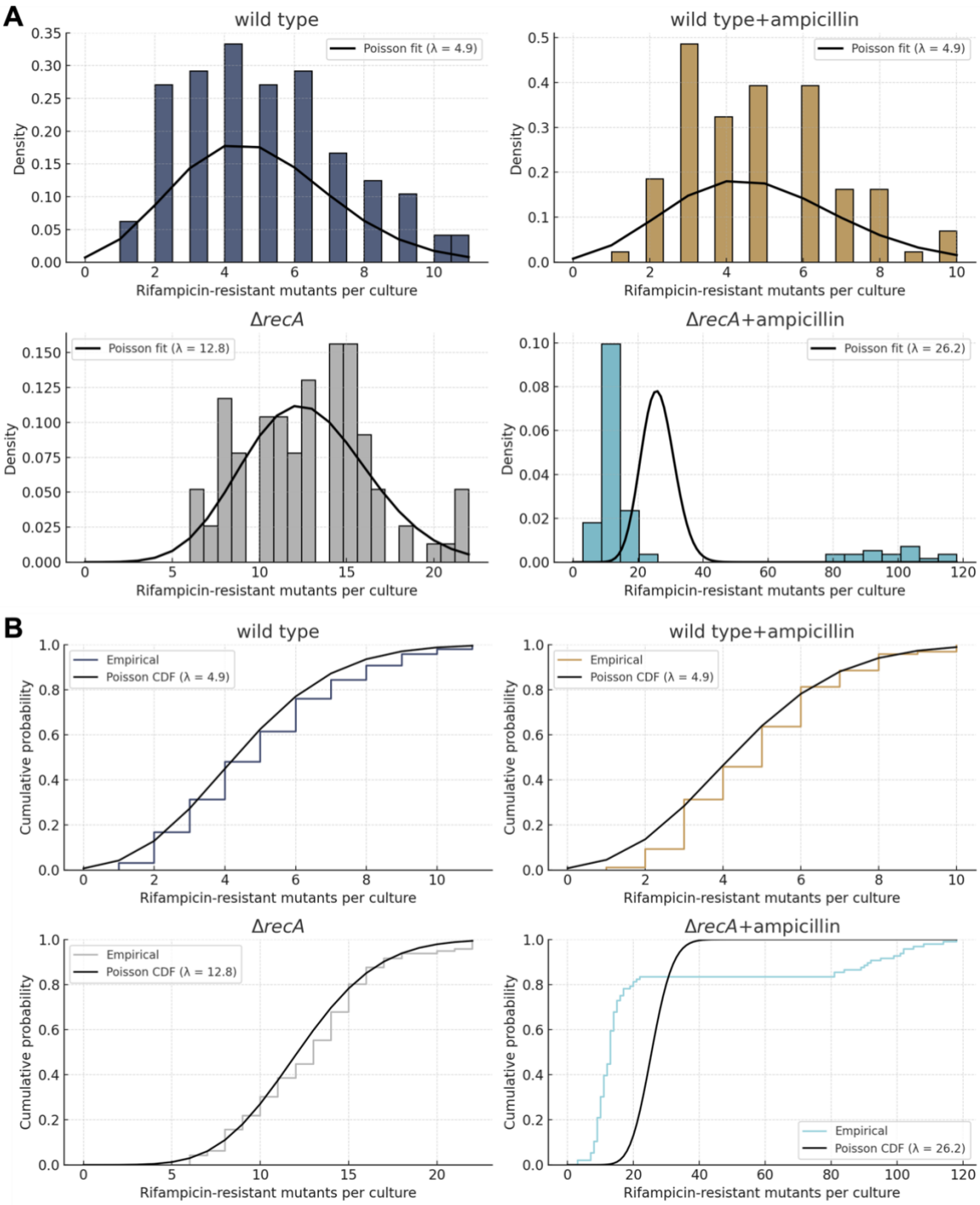
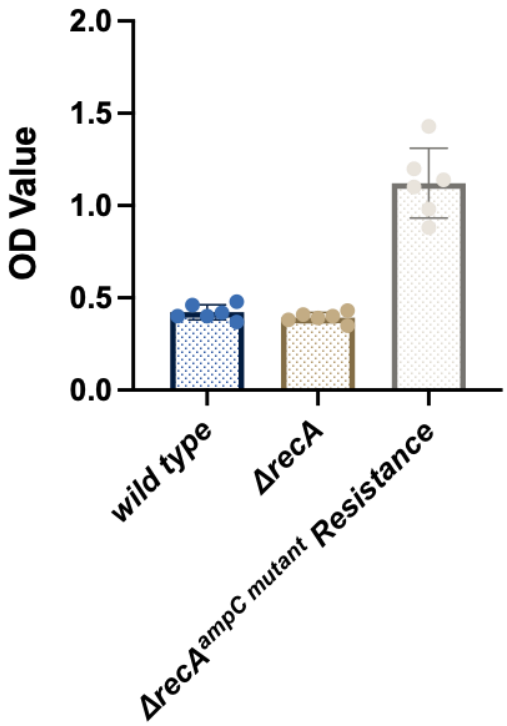


Fig. S5. Distribution fitting and fluctuation analysis support a selection-driven resistance mechanism in the *ΔrecA* strain. (A) Rifampicin-resistant mutant counts from replicate cultures were fitted to theoretical Poisson distributions based on observed means. **(B)** Fluctuation analysis using the Luria-Delbrück framework revealed long-tailed, highly skewed mutation count distributions in the *ΔrecA* strain under the treatment of ampicillin, characteristic of jackpot cultures arising from early-arising resistant mutants.



934
935 **Figure S6. The activity of β-lactamase was increased in the *ΔrecA* culture supernatants.**
936 The *ΔrecA* strain was treated with ampicillin at 50 μg/ml for 8 hours, and surviving isolates
937 harbouring the *ampC* mutations were selected. The level of β-lactamase in cell culture
938 supernatants was determined by the absorbance at OD_{490}. The levels of β-lactamase in the wild
939 type or *ΔrecA* strain culture supernatants without exposure to ampicillin were tested as a control.
940 Each experiment was independently repeated six times. Each experiment was independently
941 repeated at least six, and the data are shown as mean ± SEM.

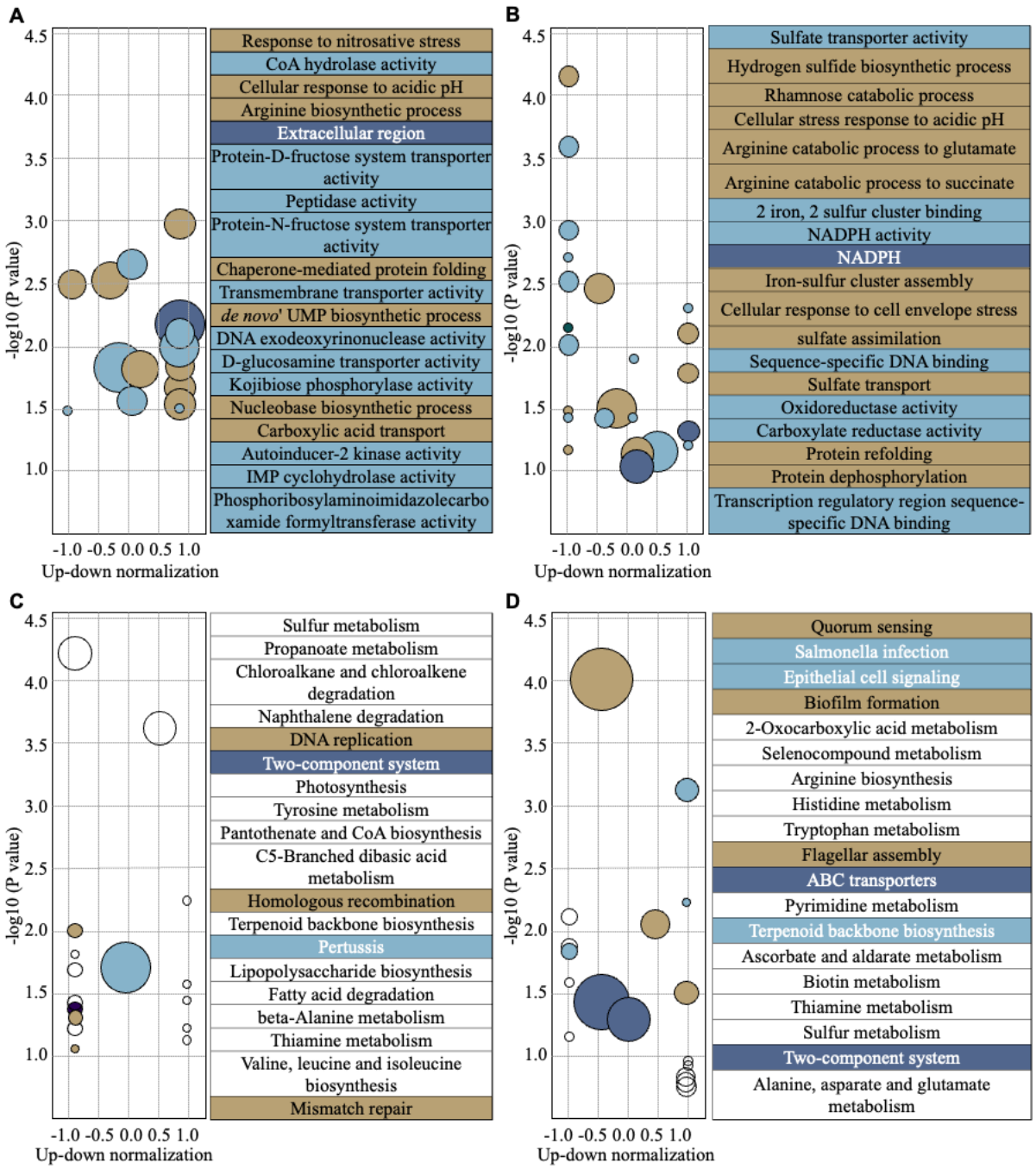


Figure S7. Transcriptional responses of the wild type and *ΔrecA* strain after single treatments with ampicillin for 8 hours. GO analysis was performed following the GOseq approach, and different genes in the wild type **(A, left)** and the *ΔrecA* strain **(B, left)** were plotted. KEGG pathway enrichment 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.