Superfast Evolution of Multi-drug Antibiotic Resistance

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In the presence of antibiotics, SOS response supports bacteria survival by activating the DNA repair system. Here, we find that the reduction of the SOS response by deletion of its master regulator *recA* can cause an even superfast establishment of antibiotic resistance (20-fold MIC) in *Escherichia coli*, which only takes 8 hours after a single exposure to ampicillin. And the gene *acrB* mutations were observed with a multi-drug resistance to other classes of antibiotics. This process is accompanied with the rapidly occurring DNA mutations, but orthogonal to the SOS response.

Homologous recombination (HR) is the process responsible for maintaining genome stability in all living organisms; it is particularly important for repairing DNA double-strand breaks, which becomes the central for cancer treatment (1). A key protein of the HR pathway in eukaryotic cells is Rad51 (2, 3). Rad51 belongs to the *recA*/*RAD51* gene family that arose from a gene duplication of the archaeal RadA protein and is highly conserved throughout evolution. Many recent findings have indicated Rad51 protein is overexpressed in a variety of tumours, and the high expression of Rad51 is related to poor prognosis (4). Therefore, HR inhibition of Rad51 may provide another mechanism of therapeutic target for the chemosensitization and radiosensitization of cancer. Some Rad51 inhibitors have being assessed in clinical trials on its

safety, tolerability and pharmacokinetics, including the drug CYT-0851 (5). However, in prokaryotic cells, RecA is the central protein that is loaded onto the ssDNA tails and forms a contiguous nucleoprotein filament and severs as a master regulator in the SOS response system (2, 3). Although RecA and Rad51 share only ~30% sequence homology, the filaments they form and the conformational changes they induce in DNA are nearly identical (6). In some studies, it is shown that the inhibitors targeting to Rad51 can strongly inhibit the activity of RecA (7). More importantly, the deficiency of DNA repair may also increase the rate of drug resistant mutagenesis induced by the antibiotic exposure. Therefore, given that the antibiotics are a critical tool for fighting infections in cancer patients who may have compromised immune systems, it is urgent to investigate whether the suppression on RecA can influence the evolution of antibiotic resistance in the clinical anti-infection therapy.

Here, we constructed a *recA* deletion *E. coli* strain (*ΔrecA*) and exposed them to a single dose of ampicillin at 50 μg/ml. Surprisingly, we observed an unexpected superfast emergence of resistance after only 8 hours single exposure to ampicillin (Fig. 1A). Genetic rescue of the *ΔrecA* strain with a plasmid encoding *recA* gene recovered its susceptibility to ampicillin, which shows this superfast resistance is dependent on the *recA* deletion (Fig. 1A). More importantly, this resistance, once being established, became stable and heritable, as after growing the culture continuously in an antibiotic-free medium for 7 days, they maintained the resistance ability to ampicillin (Fig. 1B).

We further show that this superfast evolution of antibiotic resistance already involves significant amounts of DNA mutations in the *ΔrecA* strain, which explains the stable and heritable resistance. The evolution of antibiotic resistance, from tolerance to resistance, have been reported when bacteria are exposed to antibiotics for a few weeks in *in vitro* evolutionary experiments (8) and clinical settings (9). Here, we observed the tolerance after the single exposure of 50 μg/ml ampicillin in the wild type strain (Fig. S1A-C), and the daily intermittent treatment with the same concentration of ampicillin for two weeks caused the evolution of antibiotic resistance (Fig. S1D).

For the evolution from antibiotic tolerance to resistance in the wide type strain, the whole-genome sequencing revealed that all resistant bacteria harboured mutations at the promoter of *ampC* (Fig. S1E). In comparison, in the *ΔrecA* strain, DNA mutations mainly include the mutations at the promoter of *ampC* and the gene *acrB* (Fig 1C and Table S1). The mutations at

the promoter of gene *ampC* were accompanied with the significantly increased production of β-lactamase (Fig. 1D). As the gene *acrB* mutations encode a major multi-drug efflux pump AcrB of gram-negative bacteria (10, 11), we also observed a multi-drug resistance to other classes of antibiotics, including the chloramphenicol and kanamycin in the *ΔrecA* strain (Fig. S2A and B). Treatment with 1-(1-Naphthylmethyl) piperazine (NMP), an inhibitor of the AcrB efflux pump, convinced that the gene *acrB* mutations conferred the antibiotic resistance in the gene *acrB* mutant resistant isolates, as the inhibition on AcrB restored its sensitivity to ampicillin (Fig. 1E).

Considering the potential effect of gene duplication and amplification (GDA) on the emergence of resistance in the *ΔrecA* strain (12), we further validated the chromosomal gene copy number variations (CNVs) using the whole-genome sequencing and droplet digital PCR (ddPCR) methods, but no difference was detected (Fig. S3), which suggests that GDA was not associated with this emergence of antibiotic resistance.

To further characterize this unexpected superfast evolution of antibiotic resistance, we applied a mutant prevention concentration (MPC) assay to determine the mutation frequency, and found that the single treatment of ampicillin already induced a higher mutation frequency in the *ΔrecA* strain, compared with that in the wild type strain (from 10^{-9} to 10^{-7} mutations per generation). Complementation of *recA* rescued it to a similar rate as the wild type strain (Fig. 1F). As no apparent difference of MPC was found in between the wild type and the *ΔrecA* strain without treatment (Fig. 1F), we can conclude that the mutation frequency does not naturally increase in the *ΔrecA* strain. These results characterize the repression of DNA repair induced evolution in the *ΔrecA* strain under the antibiotic exposure.

To further study the DNA repair, we applied super-resolution imaging to pinpoint the dynamic locations of the chromosome and DNA polymerase I that participates in the repair of DNA damage (13). We observed a formation of multinucleated filaments in both the wild type and the *ΔrecA* strain after the 8-hour exposure to ampicillin (Fig. 2A and B). The typical filamentation may suggest a time window for bacteria to repair the DNA damage (14). However, the expression level of DNA polymerase I was significantly suppressed in the *ΔrecA* strain (Fig. 2C and D), and the super resolution colocalization results reveal that the co-localization ratio between the chromosome and DNA polymerase I was significantly lower in the *ΔrecA* strain

compared with that in the wild type strain (Fig. 2E), suggesting the induction of DNA repair being repressed in the *ΔrecA* strain.

Because RecA is critical in the activation of SOS response that induces DNA repair, we further studied whether the superfast emergence of resistance observed in the *ΔrecA* strain has anything to do with the SOS response. To test this possibility, we used two single-gene mutants of the SOS response, *ΔlexA*, resulting in constitutive induction of the SOS response, *lexA3* where the SOS response is always switched off, and exposed them to 50 μg/ml ampicillin for 8 hours. In either case, no antibiotic resistance was observed (Fig. 2F). These results confirm that the superfast emergence of antibiotic resistance bypasses the SOS system in the *ΔrecA* strain.

In short, we observed a superfast evolution of antibiotic resistance in *Escherichia coli* once RecA, the master regulator of the SOS response, being deleted. Rapid DNA mutations, featuring at the promoter of *ampC* and the gene *acrB,* underpin the superfast emergence of antibiotic resistance as well as being resistant to other classes of antibiotics. The whole process is orthogonal to the well-known SOS response. These findings suggest that the hindrance of DNA repair not only generally antagonizes cells fitness, but also provides bacteria with genetic plasticity to adapt to diverse stressful environments and can dramatically accelerate the evolution of antibiotic resistance within only a few hours, which suggests that cares should be taken in using DNA repair inhibitor to strengthen the efficacy of antibiotics. Thereby, from a clinical perspective, our finding significantly highlights the possibility that the synergistic drug combination especially in the patients with cancer treatment, while fostering the genetic instability and enhancing the genetic diversity, may lead to an even superfast evolution of antibiotic resistance in bacteira.

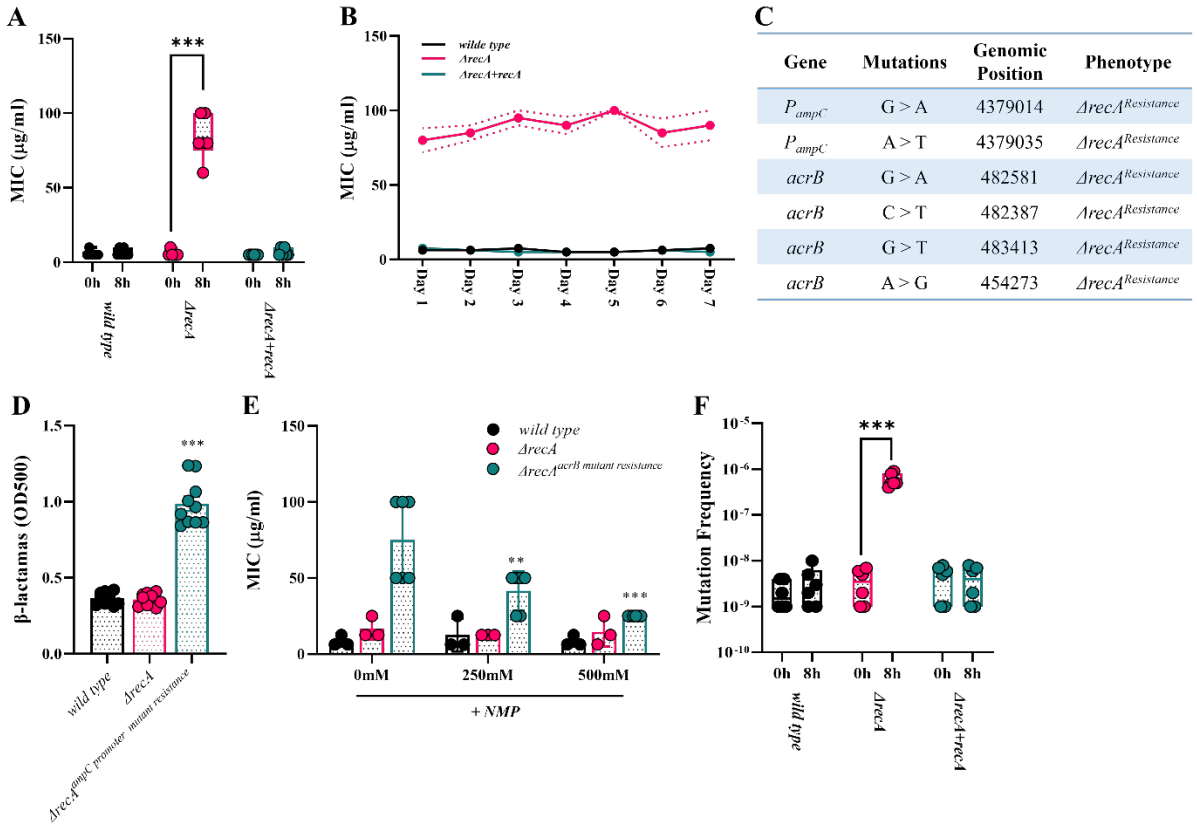


Figure 1. A superfast evolution of antibiotic resistance caused by the *recA* deletion. (A) The emergence of antibiotic resistance after a single 8-hour exposure to ampicillin (50 μg/ml) in the *ΔrecA* strain. **(B)** Heritable resistance was convinced in the *ΔrecA* resistant isolates. Surviving cells after the exposure to ampicillin were continuously cultured in antibiotic-free media for 7 days. MIC was measured in each day. **(C)** Mainly detected DNA mutations associated with the drug resistance in the *ΔrecA* resistant isolates. **(D)** Levels of β-lactamase in each strain determined by the absorbance at OD500. **(E)** The wild type, *ΔrecA*, and ampicillin exposure-induced *ΔrecA* resistant isolates were treated with NMP at 0, 250, and 500 mM for 12 hours. **(F)** The single 8-hour exposure to ampicillin (50 μg/ml) significantly increased the mutation frequency in the *ΔrecA* strain compared to that of other strains. Each experiment was independently repeated at least 4 times. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

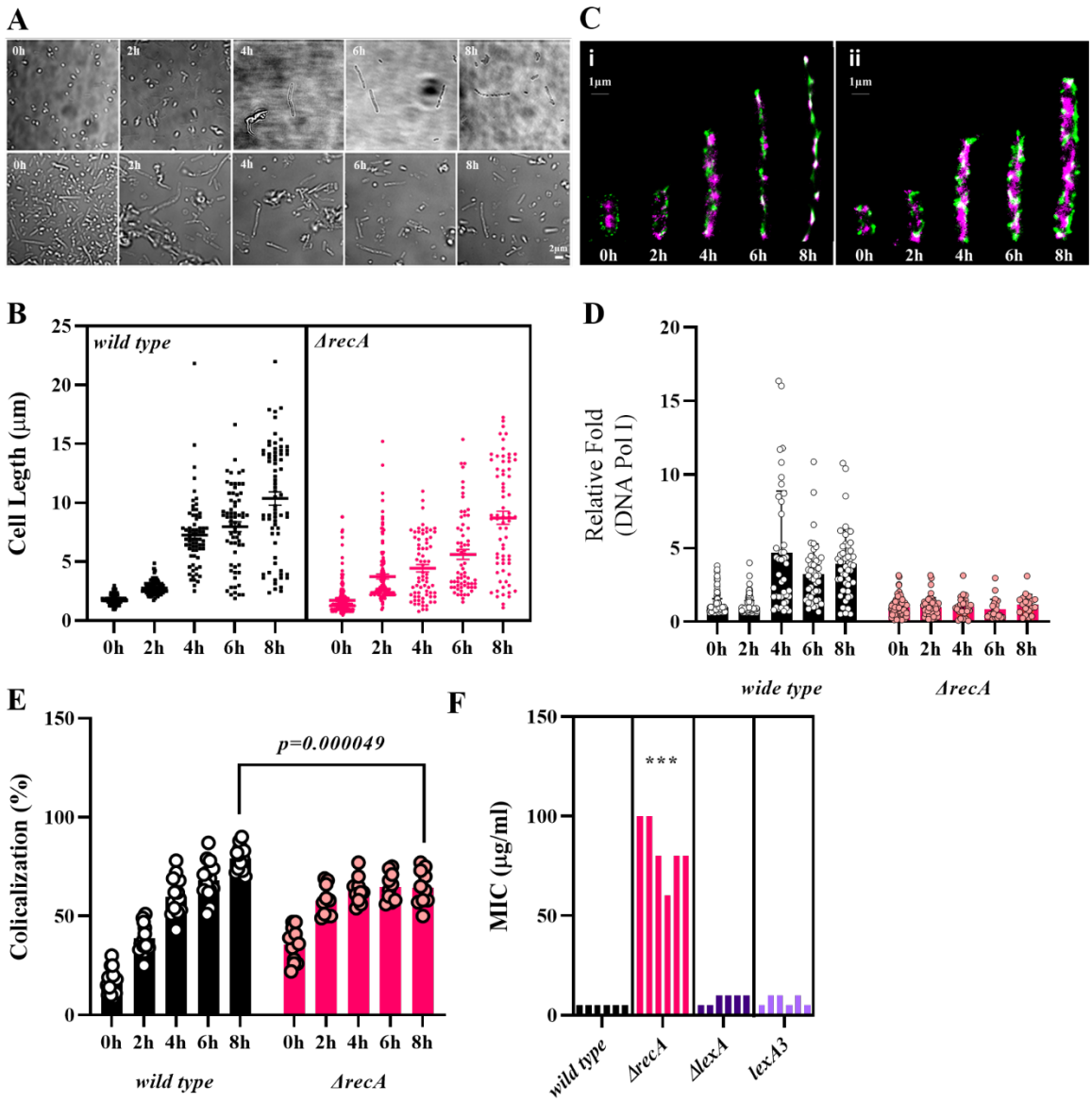


Figure 2. The superfast evolution of antibiotic resistance in the *ΔrecA* strain related to the hindrance of DNA repair but orthogonal to the SOS response. (A) After the exposure to ampicillin at 50 μg/ml for 0, 2, 4, 6, and 8 hours, surviving cells were fixed and imaged. Top, the wild type strain. Bottom, the *ΔrecA* strain. **(B)** The length of filaments counted in the wild type (n=253) and the *ΔrecA* strain (n=216). **(C)** Multinucleated filaments were observed in either (i) the wild type or (ii) the *ΔrecA* strain. Purple, *E. coli* chromosome. Green, DNA polymerase I. **(D)** The relative fold of DNA polymerase I was measured in the wild type and *ΔrecA* strain. **(E)** The co-localization between the chromosome and DNA polymerase I was statistically calculated. (E) After the exposure to ampicillin at 50 μg/ml for 8 hours, the MICs were measured in the wild type, the *ΔrecA*, the *ΔlexA*, and the *lexA3* strain. This experiment was independently repeated at least 6 times. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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 on LB agar (1.5% w/v) plates at 37ºC, unless stated otherwise, antibiotics were supplemented, where appropriate. Whenever possible, antibiotic stock solutions were prepared fresh before the use.

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, 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.

To test the capacity for tolerance, the surviving isolates were immediately used or stored at -80ºC for future use. 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 supplemented with the appropriate selective antibiotics and incubated 16 hours at 37ºC. Single colonies were isolated and used to test the resistance or stored at -80ºC for future use.

For the intermittent 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 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. The killing treatment was applied as above to the regrown culture and repeated until resistance was established.

Antibiotic susceptibility testing

The susceptibility of *E. coli* cells to antibiotics was measured by using minimum inhibitory concentration (MIC) testing (15).

The capacity of tolerance was measured by using the minimum duration for killing 99% of the population (MDK_{99}) testing (16).

ScanLag analysis

To determine the types of tolerance observed, a ScanLag analysis was applied followed by previously reported methods (17, 18).

Mutation frequency test

Bacterial population mutation frequency was evaluated based on the approach of the Delbrück-Luria Fluctuation test (19).

Transformation with plasmids

Bacterial transformation with plasmids was followed by a reported protocol using the heat shock method (20). Plasmids used in this work are listed in the Table S3.

Measurement of β-lactamase

The amount of β-lactamase was measured by the absorbance at OD500 followed by a previous reported method with modifications (21).

Construction of deletion mutants

Lambda Red recombination was used to generate various gene deletions in *E. coli* strains followed by previous reported methods with modifications (22, 23). Primers used in this work are listed in the Table S4.

DNA extraction

Chromosomal DNA was extracted and purified using the PureLink™ Genomic DNA mini kit (ThermoFisher Scientific). Plasmid DNA was extracted and purified using the PureLink™ Quick Plasmid Miniprep kit (ThermoFisher Scientific).

Whole genome sequencing

The genomic sequencing was conducted following the Nextera Flex library preparation kit process (Illumina), and processed by Sangon Biotech, Shanghai, China.

220 **Droplet digital PCR (ddPCR)**

221 Genomic DNA samples were added to the Bio-Rad 2 x ddPCR supermix at amounts of 0.05
222 ng DNA per 22 µl ddPCR reaction, according to the ddPCR Bio-Rad user manual. Primers are
223 used in this work are listed in Table S4. Samples were converted into droplets using a Bio-Rad
224 QX200 droplet generator. After the droplet generation, the plate was transferred to a thermal
225 cycler and reactions were run under the standard cycling conditions. After PCR, the plate was
226 loaded onto the Bio-Rad QX200 Droplet Digital Reader, and data analysis was performed using
227 Bio-Rad Quantasoft™ software.

228

229 **Immunofluorescence labelling**

230 To label the bacterial chromosome, a Click-iT EdU kit was used following the manufacturer's
231 instruction (ThermoFisher) and as described before (24). To label DNA polymerase I, the cells
232 were blocked and permeabilized with blocking buffer (5% wt/vol bovine serum albumin
233 [Sigma-Aldrich] and 0.5% vol/vol Triton X-100 in PBS) for 30 min and then incubated with
234 10 µg/ml primary antibody against the DNA polymerase I (ab188424, abcam) in blocking
235 buffer for 60 min at room temperature. After washing with PBS three times, the cells were
236 incubated with 2 µg/ml fluorescently labelled secondary antibody (Alexa 647, A20006,
237 ThermoFisher) against the primary antibody in blocking buffer for 40 min at room temperature.
238 After washing with PBS three times, the cells were postfixed with 4% (wt/vol)
239 paraformaldehyde in PBS for 10 min and stored in PBS before imaging.

240

241 **Super-resolution imaging and data analysis**

242 Super-resolution imaging was performed using the Stochastic Optical Reconstruction
243 Microscopy (STORM) as described previously (25, 26). STORM image analysis, drift
244 correction, image rendering, protein cluster identification and images presentation were
245 performed using Insight3[42], custom-written Matlab (2012a, MathWorks) codes, SR-Tesseler
246 (IINS, Interdisciplinary Institute for Neuroscience) (27), and Image J (National Institutes of
247 Health).

248

249 **Statistical analysis**

250 Statistical analysis was performed using GraphPad Prism v.9.0.0. All data are presented as
251 individual values and mean or mean ± s.e.m. A two-tailed unpaired Student's t-test using a 95%
252 confidence interval was used to evaluate the difference between two groups. For more than two
253 groups, a one-way ANOVA was used. 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 fourth with similar results.

Data availability

Sequence data that supports the findings of this study have been deposited in GEO repository with the GEO accession numbers GSE179434.

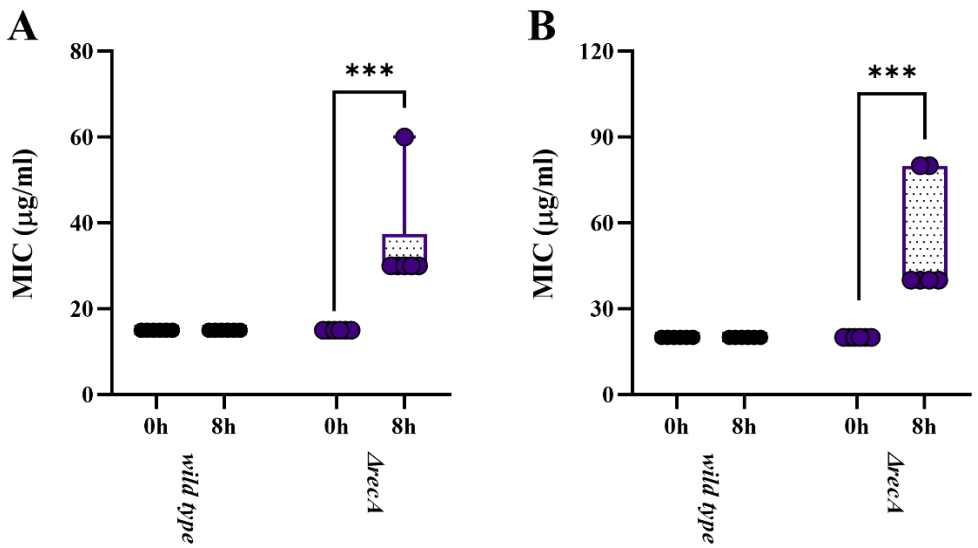


Figure S1. The multi-drug resistance to other types of antibiotics. After the exposure to ampicillin at 50 μg/ml for 8 hours, the surviving cells were tested for their resistance to other types of antibiotics including **(A)** chloramphenicol and **(B)** kanamycin. Each experiment was independently repeated 4 times. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

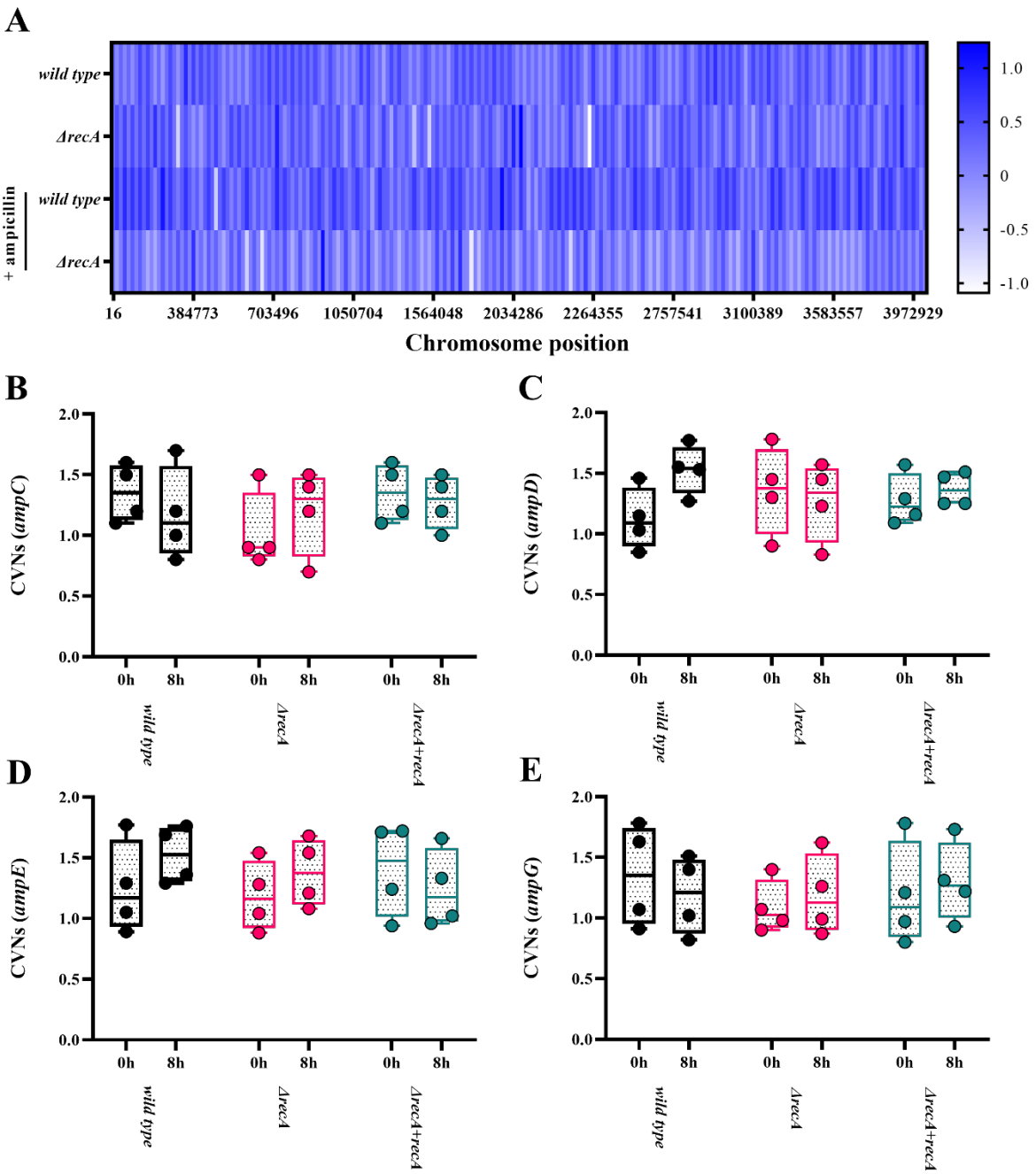
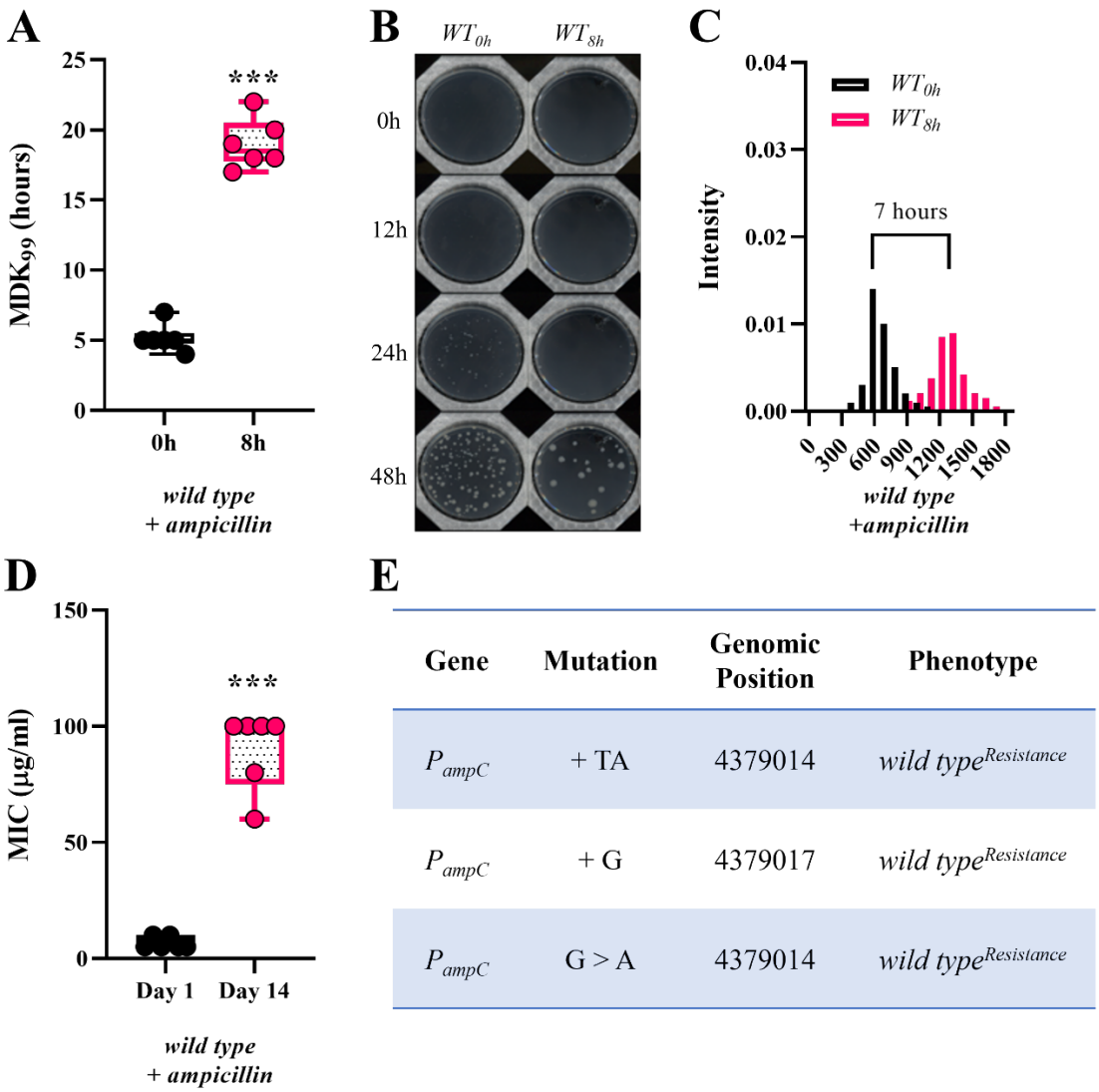


Figure S2. The copy number variations were not changed in the *ΔrecA* resistant isolates. After the treatment of ampicillin at 50 μg/ml for 8 hours, **(A)** the copy number variations of genes in chromosome were measured using the whole-genome sequencing method. The copy number variations of the ampicillin-resistance related genes in *E. coli* were verified using the ddPCR method, including **(B)** *ampC*, **(C)** *ampD*, **(D)** *ampE*, and **(E)** *ampG*. Each experiment was independently repeated 4 times.



E

Gene	Mutation	Genomic Position	Phenotype
P_{ampC}	+ TA	4379014	*wild type*^{Resistance}
P_{ampC}	+ G	4379017	*wild type*^{Resistance}
P_{ampC}	G > A	4379014	*wild type*^{Resistance}

Figure S3. The wild type strain evolved to be tolerance after a single exposure to ampicillin, but to be resistance after an intermittent treatment with ampicillin. (A) MDK_{99} of the wild type strain after the exposure to ampicillin at 50 μg/ml for 8 hours. **(B)** The type of tolerance was defined by using the ScanLag system. The appearance and morphology of each single colony were automatically monitored. **(C)** Tolerant cells had an approx. 7 hours delayed appearance time showing an emergence of tolerance by lag-phase. **(D)** MICs of the wild type strain after 14 day treatment with ampicillin at 50 μg/ml for 4 hours at each exposure cycle. **(E)** Detected DNA mutations associated with the drug resistance in the wild type resistant isolates. Each experiment was independently repeated 6 times. $* p < 0.05; ** p < 0.01; *** p < 0.001.$

289 **Table S1. Additional mutations detected in the *ΔrecA* resistant isolates.**

Gene	Mutation	Genomic Position	Phenotype
crl	G > A	257908	$\Delta recA^{Resistance}$
stfE	A>AGGTTTTCGAGAGC	1209618	$\Delta recA^{Resistance}$
rrlH	CATG > C	226298	$\Delta recA^{Resistance}$
rrsC	G > GTGTT	3942261	$\Delta recA^{Resistance}$
rrlC	A > G	3945857	$\Delta recA^{Resistance}$
araC	C > T	70434	$\Delta recA^{Resistance}$
nlpA	C > T	3839895	$\Delta recA^{Resistance}$
acnB	G > A	132324	$\Delta recA^{Resistance}$
pitA	T > A	3637975	$\Delta recA^{Resistance}$
ydfR,ydfJ	G > T	1640125	$\Delta recA^{Resistance}$
arpB	T >INDEL	1803602	$\Delta recA^{Resistance}$
ftsI	A > C	93006	$\Delta recA^{Resistance}$

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

Strain	Relevant Genotype	Parent strain	Source/technique
DH5α	-	-	Lab stock
E. coli K-12	$recA^+$ $lexA^+$	-	Lab stock
LZ101	$lexA^+$ *ΔrecA::Tet*	*K-12*	This study
$recA^{complementation}$	$lexA^+$ *ΔrecA::Tet*, pJM1071-*recA*	LZ101	This study
$recA^{OE}$	$recA^+$ $lexA^+$, pJM1071-*recA*	K-12	This study
RW1570	$recA^+$ *lexA3*	K-12	Lab stock
EAW26	$recA^+$ *ΔlexA::Cm*	K-12	Lab stock

292

293 **Table S3. Plasmids used in this study.**

Plasmid	Description	Source
pJM1071-*recA*	Gene *recA* cloned into the multiple cloning site of pJM1071, spectinomycin resistance	This study
pKD46	Helper plasmid for Lambda RED recombination	Lab stock

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Table S4. Primers used in this study.

Name	Sequence
recA-FWD	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAACAAGA CGAGAAACGAGAGAGGATGCTCAC
recA-REV	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAA AAGACGTCTAAGAAACCATTATTATCATGAC
ampC-FWD	TTACTTTACCTGGGGCTATG
ampC-REV	GTGGGATCGCTTAACTTGA
ampD-FWD	GATCGACGCATTATTCACTG
ampD-REV	CGAAAGGACATACTGGACTA
ampE-FWD	GGCGTGACTTTTTTACTGTT
ampE-REV	ACTTTACCTGCGCCAATAC
ampG-FWD	CATTGGTTTCTTCTCTCTGGT
ampG-REV	AATGGCGACTAATAACAGGA
cysG-FWD	CGAAAAACTTGAATCACTGC
cysG-REV	AATGGCTTTCTGATCGTT G
P_{ampC}*-FWD*	GTGCGATGCACAATATCGTTG
P_{ampC}*-REV*	TCCTGTTCCTGATGATCGTTC

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