Bacterial SOS-independent Superfast Evolution of Multi-Drug Resistance

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Abstract

Antibiotic resistance has been a global threat to public health. Majority of antibiotics kill bacteria by inducing the DNA damage. However, bacteria can repair DNA damage via a series of intrinsic pathways including the SOS response. The master regulator of the SOS response is RecA, which was shown to be involved in an enhanced evolution of resistance to fluoroquinolone. Until very recently, the relationship between the evolution of β-lactam resistance and the SOS response remains undefined. Here, we find a superfast evolution of β-lactam resistance (20-fold MIC) following the deletion of RecA in *Escherichia coli* and treatment with a single dose of β-lactams in 8 hours. Importantly, once this type of resistance being established, it was stable and heritable. Controversially to previous findings, our results indicate this process is completely orthogonal to the SOS response but dependent on the hindrance of DNA repair. In addition, we observe explosive appearance of drug-specific mutations of the bacterial genome in 8 hours of exposure to ampicillin, such as the *acrB* mutations which is responsible for multi-drug resistance. Together, these findings demonstrate that 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 accelerates the evolution of antibiotic resistance in DNA repair deficiency cells.

Importance

The evolution of antibiotic resistance can be induced by long-term exposure to antibiotics. However, we for the first time report a superfast evolution of multi-drug resistance induced by a single treatment with β-lactam in DNA repair deficiency *Escherichia coli*. More importantly, this type of evolutionary trajectory can cause a more rapid spread of drug-resistant bacteria in the community, because once the resistance being established, it was stable and heritable. In addition, from a clinical perspective, our finding significantly highlights the possibility that the synergistic drug combination between β-lactam and inhibitors targeting DNA repair system especially in the patients with cancer treatment can lead to a superfast evolution of multi-drug resistance.

Introduction

Antibiotic resistance is one of the top ten global public health threat. Bacterial resistance to penicillin was encountered in patients two years after massive productions and applications of the antibiotic began in 1945 (1, 2). Since then, antibiotic resistance has been reported against virtually all antibiotics developed to date (3). Misuse and overuse of antibiotics is the significant driver of increasing antibiotic resistance (4). A recent analysis estimated that in 2019, 4.95 million people died from illnesses in which bacterial resistance to antibiotics played a part and more than 1.27 million deaths were the direct result of antibiotic resistance (5).

Majority of antibiotics kill bacteria by inducing the DNA damage and repressing DNA repair in cells (6). However, before they take effect, bacteria can utilize a series of intrinsic pathways to repair DNA damage (6-8). Typically, bacteria respond to the induced stress environment via their defence system, known as the SOS response, that supports cell survival upon DNA damage (9,10). In this process, RecA, a homologous recombinase protein, serves as a master regulator of the SOS response, enabling bacteria to repair DNA damage (11-13). Therefore, studies point to the possibility of suppressing RecA activity to deactivate the SOS response and to prevent bacteria from becoming resistant to antibiotics. Recently, Mark Brynildsen et al. reported that a single exposure to fluoroquinolone, a class of antibiotic directly inducing the single- or double DNA break and activating the SOS system as a response to DNA damages, can enhance the evolution of antibiotic resistance developed from persisters in a RecA and SOS response-dependent manner (14).

68 In contrast to fluoroquinolone, β-lactam, a class of antibiotic corrupting enzymes involved in
69 cell wall biogenesis, can induce the SOS response via a DpiBA two-component signal
70 transduction system, instead of damaging the DNA structure directly (15). The evolution of
71 antibiotic resistance induced by exposure to β-lactam has been widely studied using the cyclic
72 adaptive laboratory evolution (ALE) experiment. For example, when *E. coli* are exposed
73 periodically to ampicillin after 14 to 21 rounds, drug-specific mutations appear, and the
74 emergence of antibiotic resistance can be detected (16). Importantly, mutations that arose
75 during the cyclic ALE experiments are attributed to errors associated with norm normal growth,
76 and numerous rounds of β-lactams exposure are needed to observe the evolution of antibiotic
77 resistance (17). However, the relationship between the evolution of β-lactam resistance and the
78 SOS response is still unclear, and whether the short-term exposure to β-lactam can enhance the
79 evolution of β-lactam resistance developed from persisters has not been studied yet.
80
81 **Results**
82 Here, to study the role of SOS response in bacterial evolution of β-lactam resistance, we
83 constructed a *recA* deletion *E. coli* strain (*ΔrecA*) and treated them with a single dose of
84 ampicillin at 50 μg/mL for 0, 2, 4, 6 and 8 hours. Initially, we expected a formation of persisters,
85 which was observed in the wild type strain (Fig. S1) (17); however, a superfast evolution of
86 antibiotic resistance was detected in the *ΔrecA* strain after 6 hours of treatment (Fig. 1A and
87 B). In comparison, the daily intermittent treatment of ampicillin (50 μg/ml) for three weeks
88 caused the evolution of antibiotic resistance in the wild type strain (Fig. S2). The superfast
89 evolution of antibiotic resistance was similarly observed when the *ΔrecA* strain was treated
90 with another two β-lactams for 8 hours, including the penicillin G at 1 mg/mL and carbenicillin
91 at 200 μg/mL (Fig. S3A and B).
92
93 For a clinical implication, we asked if the activity of RecA is inadvertently inhibited and once
94 this type of antibiotic resistance arises in cells, whether the susceptibility can be rescued by the
95 ending treatment with inhibitors. To answer it, we continuously cultured the *ΔrecA* resistant
96 cells in an antibiotic-free medium for 7 days after the initial treatment with ampicillin and
97 found that once resistance being established it was stable and heritable (Fig. 1C). Further, we
98 restored the expression of RecA in the *ΔrecA* strain using a plasmid encoding *recA* before the
99 ampicillin exposure and found that its susceptibility to ampicillin was fully recovered (Fig. 1D).
100 However, we found that the resistance was maintained, while the expression of RecA was
101 restored after the ampicillin exposure (Fig. 1E). Taken together, these results demonstrate that

102 the ampicillin exposure can induce the superfast evolution of antibiotic resistance in the RecA-
103 deficiency cells, and more importantly, this type of evolutionary trajectory can cause a more
104 rapid spread of drug-resistant bacteria in the community.
105
106 Antibiotic resistance can evolve through a sequential accumulation of DNA mutations (18),
107 and the antibiotic resistant levels can be gradually increased and reach the highest level after
108 around 20 days treatment with antibiotics (19). In the *ΔrecA* strain, significant amounts of drug-
109 specific and general DNA mutations appeared explosively in 8 hours of ampicillin treatment
110 (Fig. 2A and Table 1), and the drug-specific mutations were included not only at the promoter
111 of *ampC,* but at the coding region of gene *acrB* (Fig. 2A). Meanwhile, we found that, after 3
112 weeks of daily treatment with ampicillin, wild type cells evolved to resistance when mutations
113 appeared at the promoter of *ampC* (Table S1).
114
115 Gene *acrB* encodes a major multi-drug efflux pump AcrB in gram-negative bacteria (20,21).
116 We, therefore, observed a multi-drug resistance to other classes of antibiotics in the *ΔrecA*
117 strain, which was exposed to ampicillin only, including the chloramphenicol and kanamycin
118 (Fig. 2B and C). Treatment with 1-(1-Naphthylmethyl) piperazine (NMP), an inhibitor of the
119 AcrB efflux pump, convinced that *acrB* mutations conferred the antibiotic resistance, since the
120 inhibition on AcrB restored the sensitivity to ampicillin in *ΔrecA* resistance isolates (Fig. 2D).
121 Additionally, mutations at the promoter of *ampC* were accompanied by the significantly
122 increased production of β-lactamase (Fig. S4), which is the most common cause of bacterial
123 resistance to β-lactam.
124
125 Considering the potential effect of gene duplication and amplification (GDA) on the emergence
126 of resistance in the *ΔrecA* strain (22), we further validated the chromosomal gene copy number
127 variations (CNVs) using whole-genome sequencing (Fig. 2E) and droplet digital PCR (ddPCR)
128 methods (Fig. S5), but there was no difference between the wild type and *ΔrecA* strain.
129
130 We next applied a mutant prevention concentration (MPC) assay to determine the mutation
131 frequency. We found that, after the single exposure to ampicillin, the mutation frequency was
132 significantly increased (from 10^{-9} to 10^{-7} mutations per generation) in the *ΔrecA* strain.
133 Conversely, the mutation frequency was not changed naturally either in the wild type or in the
134 *ΔrecA* strain without the exposure to ampicillin (Fig. 2F), which evidenced that the rapid

135 acquirement of drug-specific mutations in *ΔrecA* strain was resulted from the single treatment
136 with ampicillin in the deficiency of RecA.
137
138 Loss of efficient DNA damage repair can lead to the accelerated accumulation of DNA
139 mutations, and the trade-off between bacterial susceptibility and resistance is often driven by
140 the cellular response to DNA damage, including the SOS response. Indeed, in the *ΔrecA* strain,
141 we observed DNA mutations on the *ftsI* gene, of which inactivation products have been shown
142 to induce SOS response in *E. coli* through the DpiBA two-component signal transduction
143 system (Fig. 2A and Table 1) (15). In addition, we applied super-resolution imaging to pinpoint
144 the dynamic locations of the chromosome and DNA polymerase I, which participates in the
145 repair of DNA damage (23). During the period of 8 hours of exposure, we found a formation
146 of multinucleated filaments either in the wild type or the *ΔrecA* strain (Fig. 3A-C), which
147 suggested a time window for bacteria to repair the DNA damage (24). However, the expression
148 level of DNA polymerase I was significantly suppressed in the *ΔrecA* strain (Fig. 3D), and
149 more importantly, the super-resolution colocalization results revealed a significantly lower
150 ratio of the co-localization between the chromosome and DNA polymerase I in the *ΔrecA* strain
151 (Fig. 3E). To confirm whether the inhibition of SOS response caused the superfast evolution
152 of antibiotic resistance, we used a single-gene mutant of the SOS response, *lexA3* where the
153 SOS response is always switched off, to be treated by the single exposure to ampicillin.
154 Interestingly, antibiotic resistance was not observed in this strain (Fig. 3F). Collectively, these
155 findings indicated that the DNA repair was significantly hindered in the *ΔrecA* strain, but the
156 superfast evolution of antibiotic resistance was developed in an SOS-independent manner in
157 RecA-deficiency cells.
158
159 **Discussions**
160 SOS response is widely studied to be involved, while the RecA contributes to the evolution of
161 antibiotic resistance (25). However, we unexpectedly observed an SOS-independent evolution
162 of antibiotic resistance in *E. coli* treated with a single exposure to ampicillin for 8 hours, once
163 the RecA was deleted in cells (Fig. 1 and 3F). This finding debates the conventional option that
164 inactivation of RecA to inhibit the SOS response can prevent bacteria from becoming resistant
165 to antibiotics. Meanwhile, rapid DNA mutations appeared after 8 hours of ampicillin treatment,
166 featuring at the *acrB* and the promoter of *ampC,* underpin the superfast emergence of antibiotic
167 resistance (Fig. 2A), and more importantly, they consequently cause multi-drug resistance to
168 other classes of antibiotics (Fig. 2B and C). These findings demonstrate that the hindrance of

169 DNA repair not only generally antagonizes cells fitness (Fig. 3), but also provides bacteria with
170 genetic plasticity to adapt to diverse stressful environments and can dramatically accelerates
171 the evolution of antibiotic resistance in RecA-deficiency cells.
172
173 Recently, a paradoxal β-lactam phenotype have been reported, of which mechanism is not
174 linked to β-lactamase acquisition but associated to the higher expression of efflux pump, since
175 these isolates are less susceptible to cefoxitin but susceptible to both amoxicillin/clavulanic
176 acid (26). To our knowledge, this research is the first to show the superfast accumulation of
177 *acrB* mutations caused by a single exposure to ampicillin in RecA-deficiency cells, which
178 provides a theoretical basis for the clinical phenomena. In addition, we find that the AcrB
179 inhibitor can restore the sensitivity to β-lactam in *ΔrecA* resistance cells (Fig. 2D), which
180 creates a novel clinical strategy to combat antibiotic resistance in anti-infection therapy.
181
182 Many anti-cancer drugs target DNA repair to kill tumour cells by damaging DNA structure,
183 and the homologous recombination (HR) is the process responsible for DNA repair (27). A key
184 protein of the HR pathway in eukaryotic cells is Rad51, which belongs to the *recA*/*RAD51* gene
185 family (28,29). A series of Rad51 inhibitors is being assessed in clinical trials, but some studies
186 have shown that inhibitors targeting to Rad51 can strongly inhibit the activity of RecA, as even
187 though the RecA and Rad51 share only ~30% sequence homology, filaments they form and
188 the conformational changes they induce in DNA are nearly identical (30). Besides, inhibitors
189 of RecA have been identified to enhance the antimicrobial effects of some antibiotics, including
190 fluoroquinolone (31,32). Therefore, from a clinical perspective, our finding significantly
191 highlights the possibility that the synergistic drug combination between β-lactam and RecA
192 inhibitors especially in the patients with cancer treatment can lead to a superfast evolution of
193 β-lactam resistance, and more importantly, this type of resistant cells can rapidly spread in the
194 community and become multi-drug resistant to other types of antibiotics.
195
196 **Materials and Methods**
197 **Bacterial strains, medium and antibiotics**
198 Bacterial strains and plasmids used in this work are described in Table S2 and Table S3. Luria-
199 Bertani (LB) was used as broth or in agar plates. *E. coli* cells were grown on LB agar (1.5%
200 w/v) plates at 37ºC, unless stated otherwise, antibiotics were supplemented, where appropriate.
201 Whenever possible, antibiotic stock solutions were prepared fresh before the use.
202

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

204 For the single exposure to antibiotic experiment, an overnight culture (0.6 mL; 1 x 10^9 CFU/mL
205 cells) was diluted 1:50 into 30 mL LB medium supplemented with antibiotics (50 μg/mL
206 ampicillin, 1 mg/mL penicillin G, or 200 μg/mL carbenicillin) and incubated at 37ºC with
207 shaking at 250 rpm for 0, 2, 4, 6 and 8 hours, respectively. After each treatment, the antibiotic-
208 containing medium was removed by washing twice (20 min centrifugation at 1500 g) in fresh
209 LB medium.

210

211 To test the capacity for tolerance, surviving isolates were immediately used or stored at -80ºC
212 for future use. To test resistance, the surviving isolates were first resuspended in 30 mL LB
213 medium and grown overnight at 37ºC with shaking at 250 rpm. The regrown culture was then
214 plated onto LB agar supplemented with the appropriate selective antibiotics and incubated 16
215 hours at 37ºC. Single colonies were isolated and used to test the resistance or stored at -80ºC
216 for future use.

217

218 For the intermittent antibiotic treatment experiments, an overnight culture (0.6 mL; 1 x 10^9
219 CFU/mL cells) was diluted 1:50 into 30 mL LB medium supplemented with 50 μg/mL
220 ampicillin and incubated at 37ºC with shaking at 250 rpm for 4 hours. After treatment, the
221 antibiotic-containing medium was removed by washing twice (20 min centrifugation at 1500
222 g) in fresh LB medium. The surviving isolates were resuspended in 30 mL LB medium and
223 grown overnight at 37ºC with shaking at 250 rpm. The killing treatment was applied as above
224 to the regrown culture and repeated until resistance was established.

225

226 **Antibiotic susceptibility testing**

227 The susceptibility of *E. coli* cells to antibiotics was measured by using minimum inhibitory
228 concentration (MIC) testing (33). In brief, diluted overnight bacterial culture (10^7 CFU/mL,
229 determined by CFU counting) was used to inoculate wells of a sterile 96-well flat-bottomed
230 plate. Various concentrations of antibiotics were added to the designated wells by serial
231 dilutions with LB media to a final volume of 150 μL. Untreated controls were also included.
232 The plate was incubated in the Synergy HT BioTek plate reader (BioTek Instruments Inc.,
233 USA) at 37°C with continuous moderate shaking to prevent biofilm formation (1800 rpm, amp.
234 0.549 mm x-axis) for 24 h and was programmed to measure the OD hourly at 595 nm (Gen5
235 software, BioTek Instruments Inc., USA). The MIC was defined as the lowest concentration of

236 antimicrobial agent that inhibited 99% growth of *E. coli* when compared to the untreated
237 control.
238
239 The capacity of tolerance was measured by using the minimum duration for killing 99% of the
240 population (MDK_{99}) testing (34). After the treatment with antibiotics, surviving cells were
241 loaded in a 96 wells plate (approx. 10^4 bacteria/well) that was filled with fresh LB medium
242 supplemented with increasing amounts of ampicillin in which the lowest concentration of
243 ampicillin was 100 µg/mL. Inoculation times are set so that all rows end their respective
244 treatment at the same time. Once incubation is concluded, the plate is spun down to terminate
245 the antibiotic exposure by washing away antibiotic remains and resuspending in fresh medium.
246 The plate is then returned for overnight incubation. Empty wells indicate killing of >99% of
247 the population, because growth in the well would imply that at least one bacterium. An
248 evaluation of MDK_{99} can be read directly from the plate depending on the treatment duration
249 at which the plateau forms.
250
251 **ScanLag analysis**
252 To determine the types of tolerance observed, a ScanLag analysis was applied followed by
253 previously reported methods (35, 36). In brief, after the treatment with antibiotics, surviving
254 isolates were diluted serially and plated on the LB agar medium. The plates were placed in the
255 ScanLag setup at 37°C. The ScanLag setup is an array of office scanners, which takes images
256 of the plates automatically every 15 minutes, and thus monitors the appearance of thousands of
257 colonies. An automated image analysis application extracts the distribution of appearance time
258 of the colonies.
259
260 **Mutation frequency test**
261 Bacterial population mutation frequency was evaluated based on the approach of the Delbrück-
262 Luria Fluctuation test (37). In brief, overnight bacterial cultures were diluted 1:50 in parallel
263 cultures and plated on 50 µg/mL ampicillin to quantify the number of mutants and serially
264 diluted and plated on LB agar medium to quantify total viable cells. Colonies were quantified
265 after overnight incubation at 37°C. Mutation frequency was determined by taking the ratio of
266 ampicillin resistant colonies to the viable cell count for each sample.
267
268 **Transformation with plasmids**

269 Bacterial transformation with plasmids was followed by a reported protocol using the heat
270 shock method (38). To prepare the competent cells, *E. coli* was grown overnight in LB at 37°C
271 with shaking at 250 rpm. The following day, cultures were diluted to OD_{600} of 0.05 in 500 mL
272 of LB. Cells were grown to an OD_{600} ranging from 0.6 to 0.8, after which they were chilled on
273 ice for 10 minutes. Cells were centrifuged at 3,000 g at 4°C for 10 minutes and the supernatant
274 discarded. The pellet was resuspended in 10 mL ice cold 0.05 M $CaCl_2$ and incubated on ice
275 for 30 min. The solution was centrifuged again at 3,000 g for 10 minutes at 4°C. The pellet was
276 resuspended in 4 mL of ice cold sterile 15% glycerol (v/v) containing 0.05 M $CaCl_2$ and
277 incubated on ice for 10 min. A concentration of 50 ng (unless otherwise stated) of desired
278 plasmid was added to 50 µL of competent cells and incubated on ice for 30 min. This was then
279 heated shock by placing the tube into a 42°C water bath for 90 seconds, and following to put
280 the tubes back on ice for 5 min. After the transformation, the cells were transferred into 1 mL
281 LB medium to recover for 1 hour at 37°C with shaking at 250 rpm. 100µL undiluted cells were
282 plated out onto antibiotic selective LB agar plates. The remainder of the sample was
283 concentrated by 10 times and plated onto another plate. Plates were incubated overnight at
284 37°C (unless otherwise stated).
285
286 **Construction of deletion mutants**
287 Lambda Red recombination was used to generate various gene deletions in *E. coli* strains
288 followed by previous reported methods with modifications (39, 40). Genomic DNA of *E. coli*
289 K-12, containing insertions of a tetracycline or chloramphenicol resistance cassette to replace
290 the open reading frame of *recA*, was used to make subsequent gene deletions. Primers (Table
291 S4) were designed approximately 50 bp upstream and downstream to genes of interest on the
292 chromosome, in order to amplify the tetracycline or chloramphenicol cassette as well as the
293 flanking DNA sequence needed for homologous recombination. Phusion polymerase (NEB)
294 was used to amplify DNA sequence (Table S4) and the reaction was cleaned up using a
295 PureLink™ PCR purification kit (ThermoFisher Scientific) as per the manufacturer's
296 instructions. Background *E. coli* was made electro-competent and transformed with
297 recombinase plasmid pKD46 and selected on LB agar plates containing 100 µg/mL ampicillin
298 at 30°C. The strain now containing the plasmid was made electro-competent again using LB
299 media containing 100 µg/mL ampicillin and 0.2% arabinose at 30°C. Amplified DNA was
300 transformed into recipient strain by using 50 ng of DNA and 50µL of competent cells. Cells
301 were allowed to recover in LB media for 1 hour at 30°C. Transformation was plated onto LB
302 agar plates containing 10 µg/mL tetracycline or 17 µg/mL chloramphenicol and incubated

303 overnight at 37°C. PCR was used to confirm insertion of the tetracycline or chloramphenicol
304 resistance cassette at the correct site on the chromosome using primers upstream and
305 downstream to the gene of interest. The newly constructed mutant strains were cured of plasmid
306 pKD46 through incubation of LB streak plates at 42°C overnight. Loss of the plasmid was
307 confirmed by lack of ampicillin sensitivity on LB agar plates. Mutant strains were made
308 electro-competent and 50 µL of cells were transformed with plasmid pCP20 and incubated on
309 100 µg/mL ampicillin plates at 30°C overnight. A few colonies were then restreaked onto LB
310 plates and incubated overnight at 42°C. Loss of cassette and plasmid was confirmed by PCR
311 products.
312
313 **Measurement of β-lactamase**
314 The amount of β-lactamase was measured followed by a previous reported method with
315 modifications (41). Briefly, β-lactamase activity was assessed spectrophotometrically by
316 hydrolysis of nitrocefin. The assay mixture contained 83 µg of nitrocefin, 167 µg of BSA, 10%
317 glycerol and 0.33 mL (0.6 µg/mL of protein) of cell lysate that contained β-lactamase in a final
318 volume of 1.5 mL of 50 mM phosphate buffer. β-Lactamase activity was monitored by
319 measuring the decrease in absorbance at 390 nm for 10 min at 28°C. The enzyme activity was
320 expressed as µmol of nitrocefin hydrolysed/min/mg of protein, and the calculation was based
321 upon the molar extinction coefficient of 15 000 $M^{-1}cm^{-1}$ for nitrocefin.
322
323 **DNA extraction**
324 Chromosomal DNA was extracted and purified using the PureLink™ Genomic DNA mini kit
325 (ThermoFisher Scientific). In summary, a volume of 1 mL of overnight culture of the required
326 strain was centrifuged for 2 minutes at 10,000 g. The cell pellet was resuspended in 180 µL
327 PureLink™ genomic digestion buffer and 20 µL of Proteinase K and incubated at 55ºC for 60
328 minutes until lysis was complete. A volume of 20 µL of RNase A (provided with the kit) was
329 added to the lysate and mixed for 10 seconds on the vortex. The sample was then incubated for
330 2 minutes at room temperature. A volume of 200 µL of PureLink™ genomic Lysis/Binding
331 buffer was added to the sample and mixed for 20 seconds by vertexing until a homogenous
332 solution was obtained. 200 µL of 100% ethanol was added to the lysate and mixed well by
333 vortexing for 5 seconds. The lysate (approximately 650 µL) was loaded onto a PureLink™ spin
334 column in a collection tube and centrifuged at 10,000 g for 1 minute at room temperature. The
335 flow through liquid was discarded and the column was washed twice with wash buffer 1 and

336 wash buffer 2. The column was dried with a final spin for 3 minutes at 10,000 g. DNA was
337 eluted using 200 µL of PureLink™ genomic elution buffer into a new 1.5 mL Eppendorf tube.
338
339 Plasmid DNA was extracted and purified using the PureLink™ Quick Plasmid Miniprep kit
340 (ThermoFisher Scientific). In summary, 3 mL of overnight culture of *E. coli* harbouring desired
341 plasmid was centrifuged at 10,000 g. Cell pellet was resuspended with 250 µL of resuspension
342 buffer. Cells were lysed by adding 250 µL of lysis buffer and mixing by gently inverting the
343 tube. A volume of 350 µL of the precipitation buffer was added and sample mixed by inverting.
344 The precipitate was separated from the supernatant via centrifugation at 10,000 g for 10
345 minutes. The supernatant was loaded onto a spin column and spun for 1 minute at 10,000 g.
346 The column was washed using 700 µL of the wash buffer and spun at 10,000 g for 1 minute.
347 The column was dried, and excess wash buffer removed with a 2 minute spin at 10,000 g.
348 Plasmid DNA was eluted using 50 µL of TE buffer into a new 1.5 mL Eppendorf tube.
349
350 **Whole genome sequencing**
351 The genomic sequencing was conducted following the Nextera Flex library preparation kit
352 process (Illumina), and processed by Sangon Biotech, Shanghai, China. Briefly, genomic DNA
353 was quantitatively assessed using Quant-iT picogreen dsDNA assay kit (Invitrogen, USA). The
354 sample was normalised to the concentration of 1 ng/µL. 10 ng of DNA was used for library
355 preparation. After tagmentation, the tagmented DNA was amplified using the facility's custom
356 designed i7 or i5 barcodes, with 12 cycles of PCR. The quality control for the samples was
357 done by sequencing a pool of samples using MiSeq V2 nano kit - 300 cycles. After library
358 amplification, 3 µL of each library was pooled into a library pool. The pool is then clean up
359 using SPRI beads following the Nextera Flex clean up and size selection protocol. The pool
360 was then sequenced using MiSeq V2 nano kit (Illumina, USA). Based on the sequencing data
361 generated, the read count for each sample was used to identify the failed libraries (i.e., libraries
362 with less than 100 reads). Moreover, based on the read count, libraries were pooled at a
363 different amount to ensure equal representation in the final pool. The final pool was sequenced
364 on Illumina NovaSeq 6000 Xp S4 lane, 2 × 150 bp.
365
366 **Droplet digital PCR (ddPCR)**
367 Genomic DNA samples were added to the Bio-Rad 2 x ddPCR supermix at amounts of 0.05
368 ng DNA per 22 µL ddPCR reaction, according to the ddPCR Bio-Rad user manual. Samples
369 were converted into droplets using a Bio-Rad QX200 droplet generator. After the droplet

370 generation, the plate was transferred to a thermal cycler and reactions were run under the
371 standard cycling conditions. After PCR, the plate was loaded onto the Bio-Rad QX200 Droplet
372 Digital Reader, and data analysis was performed using Bio-Rad Quantasoft^{TM} software. CNV
373 analysis by ddPCR involves quantification of target and reference loci through the use of
374 duplex target and reference assays. In QuantaSoftTM software, copy number is determined by
375 calculating the ratio of the target DNA concentration to the reference DNA concentration, times
376 the number of copies of reference species in the genome. The error bars on a CN estimate in
377 QuantaSoftTM software are the 95% confidence interval of this measurement.
378
379 **Super-resolution imaging and data analysis**
380 Super-resolution imaging was performed using the Stochastic Optical Reconstruction
381 Microscopy (STORM) as described previously (42-44). In summary, a 35 mm cell culture dish
382 (0.17 mm) was placed in a large beaker (keep the dish upright and straight) for the rinsing once
383 with the ultrapure water MilliQ. 1 M KOH was added into the beaker that is then placed in an
384 ultrasonic cleaning instrument for washing 30 minutes. Using MilliQ water to rinse the culture
385 dish for three times. Then, the dish was placed back to the ultrasonic cleaning instrument for
386 the secondary washing following with the same protocol. After the washing, ultrapure water
387 was discarded, and the dish was dry using high-purity nitrogen and UV exposure for 30 minutes.
388 Dishes were stored in a cool and dry place until the use. Cells were fixed with NaPO4 (30 nM),
389 formaldehyde (2.4%), glutaraldehyde (0.04%). Samples were incubated at room temperature
390 for 15 minutes followed by 45 minutes on ice. Samples were then centrifuged to pellet cells
391 and the supernatant discarded. Cell pellets were washed twice with phosphate-buffered saline
392 (PBS), pH 7.4. Cells were then resuspended in 200 µL of GTE buffer. Samples were kept on
393 ice until the next step. To label the bacterial chromosome, a Click-iT EdU kit was used
394 following the manufacturer's instruction (ThermoFisher) and as described before (42). To label
395 DNA polymerase I, the cells were blocked and permeabilized with blocking buffer (5% wt/vol
396 bovine serum albumin [Sigma-Aldrich] and 0.5% vol/vol Triton X-100 in PBS) for 30 min and
397 then incubated with 10 µg/mL primary antibody against the DNA polymerase I (ab188424,
398 abcam) in blocking buffer for 60 min at room temperature. After washing with PBS three times,
399 the cells were incubated with 2 µg/mL fluorescently labelled secondary antibody (Alexa 647,
400 A20006, ThermoFisher) against the primary antibody in blocking buffer for 40 min at room
401 temperature. After washing with PBS three times, the cells were postfixed with 4% (wt/vol)
402 paraformaldehyde in PBS for 10 min and stored in PBS before imaging. STORM image
403 analysis, drift correction, image rendering, protein cluster identification and images

presentation were performed using Insight3[42], custom-written Matlab (2012a, MathWorks) codes, SR-Tesseler (IINS, Interdisciplinary Institute for Neuroscience) (45), 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 ± s.e.m. A one-tailed unpaired Student's t-test using a 95% confidence interval was used to evaluate the difference between two groups. For more than two 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.

Reference

1. Aminov RI. A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol*. **1**, 134 (2010).
2. Hamilton KC. The Creation of Antibiotics and the Birth of Modern Medicine. *Emerging Infect Dis*. **25**, 196 (2019).
3. Ventola CL. The antibiotic resistance crisis causes and threats. *P & T*. **40**, 277-283 (2015).
4. Goossens H, Ferech M, Vander Stichele R, Elseviers M, ESAC Project Group. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *The Lancet*. **365**, 579-587 (2015).
5. Antimicrobial Resistance Collaborators. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*. **399** (10325), 629-655 (2019).
6. Rebecca S. Shapiro. Antimicrobial-Induced DNA Damage and Genomic Instability in Microbial Pathogens. *PLOS Pathogens*. **11**, e1004678 (2015).
7. Darja Žgur-Bertok. DNA Damage Repair and Bacterial Pathogens. *PLOS Pathogens.* **9** *PLOS Pathogens*, e1003711 (2013).
8. Dale B. Wigley. Bacterial DNA Repair: Recent Insights into The Mechanism of RecBCD, AddAB and AdnAB. *Nature Reviews Microbiology*. **11**, 9-13 (2013).

437 9. Alexander Harms, Etienne Maisonneuve, Kenn Gerdes. Mechanisms of Bacterial
438 Persistence during Stress and Antibiotic Exposure. *Science*. **354**, aaf4268 (2016).
439 10. Katarzyna H. Maslowska, Karolina Makiela-Dzbenska, Iwona J. Fijalkowska. The SOS
440 System: A Complex and Tightly Regulated Response to DNA Damage. *Environ Mol*
441 *Mutagen.* **60**, 368-384 (2019).
442 11. Jason C. Bell, Stephen C. Kowalczykowski. RecA: Regulation and Mechanism of a
443 Molecular Search Engine. *Trends in Biochemical Sciences*. **41**, 491-507 (2016).
444 12. Michael M. Cox. Motoring Along with the Bacterial RecA Protein. *Nature Reviews*
445 *Molecular Cell Biology*. **8**, 127-138 (2007).
446 13. Michael M. Cox. The Bacterial RecA Protein as a Motor Protein. *Annu. Rev. Microbiol.* **57**,
447 551-577 (2003).
448 14. Barrett, T.C., Mok, W.W.K., Murawski, A.M. et al. Enhanced antibiotic resistance
449 development from fluoroquinolone persisters after a single exposure to antibiotic. *Nat*
450 *Commun*. **10**, 1177 (2019).
451 15. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN. SOS response
452 induction by beta-lactams and bacterial defense against antibiotic lethality. *Science*.
453 **305**(5690), 1629-31 (2004).
454 16. Jahn LJ, Munck C, Ellabaan MMH, Sommer MOA. Adaptive Laboratory Evolution of
455 Antibiotic Resistance Using Different Selection Regimes Lead to Similar Phenotypes and
456 Genotypes. *Front Microbiol*. **8**:816 (2017).
457 17. Irit Levin-Reisman et al. Antibiotic Tolerance Facilitates the Evolution of Resistance.
458 *Science*. **355**, 826-830 (2017).
459 18. Weinreich DM, Delaney NF, Depristo MA, Hartl DL. Darwinian evolution can follow only
460 very few mutational paths to fitter proteins. *Science*. **312**(5770), 111-4 (2006).
461 19. Toprak E, Veres A, Michel JB, Chait R, Hartl DL, Kishony R. Evolutionary paths to
462 antibiotic resistance under dynamically sustained drug selection. *Nat Genet*. **44**(1), 101-5
463 (2011).
464 20. Jessica M. A. Blair et al. Molecular Mechanisms of Antibiotic Resistance. *Nature Reviews*
465 *Microbiology*. **13**, 42-51 (2015).
466 21. Imane El Meouche, Mary J. Dunlop. Heterogeneity in Efflux Pump Expression Predisposes
467 Antibiotic-Resistant Cells to Mutation. *Science*. **362**, 686-690 (2018).
468 22. Linus Sandegren, Dan I. Andersson. Bacterial Gene Amplification: Implications for the
469 Evolution of Antibiotic Resistance. *Nature Reviews Microbiology*. **7**, 578-588 (2009).

470 23. Marilyn Monk, Michael Peacey, Julian D. Gross. Repair of Damage Induced By Ultraviolet
471 Light in DNA Polymerase-Defective *Escherichia Coli* Cells. *Journal of Molecular Biology*.
472 **58**, 623-624 (1971).
473 24. Sheryl S Justice, David A Hunstad, Lynette Cegelski, Scott J Hultgren. Morphological
474 Plasticity as a Bacterial Survival Strategy. *Nature Review Microbiology*. **6**, 162-168 (2008).
475 25. Athanasia Pavlopoulou. RecA: A Universal Drug Target in Pathogenic Bacteria. *Frontiers*
476 *in Bioscience*. **23**, 36-42 (2018).
477 26. Pages JM, Lavigne JP, Leflon-Guibout V, Marcon E, Bert F, Noussair L, Nicolas-Chanoine
478 MH. Efflux pump, the masked side of beta-lactam resistance in Klebsiella pneumoniae
479 clinical isolates. *PLoS One*. 4(3), e4817 (2009).
480 27. Xuan Li and Wolf-Dietrich Heyer. Homologous recombination in DNA repair and DNA
481 damage tolerance. *Cell Research*. **18**, 99-113 (2008).
482 28. PR Bianco, RB Tracy, SC Kowalczykowski. DNA strand exchange proteins: a biochemical
483 and physical comparison. *Front Biosci.* **3**, D570-603 (1998).
484 29. P Sung, L Krejci, S Van Komen, MG Sehorn, Rad51 recombinase and recombination
485 mediators. *J Biol Chem.* 278, 42729-42732 (2003).
486 30. T Ogawa, X Yu, A Shinohara, EH Egelman, Similarity of the yeast RAD51 filament to the
487 bacterial RecA filament. *Science* **259**, 1896-1899 (1993).
488 31. Wigle TJ, Sexton JZ, Gromova AV, Hadimani MB, Hughes MA, Smith GR, Yeh LA,
489 Singleton SF. Inhibitors of RecA activity discovered by high-throughput screening: cell-
490 permeable small molecules attenuate the SOS response in Escherichia coli. *J Biomol Screen*.
491 **14**(9), 1092-101 (2009).
492 32. Yakimov A, Pobegalov G, Bakhlanova I, Khodorkovskii M, Petukhov M, Baitin D.
493 Blocking the RecA activity and SOS-response in bacteria with a short α-helical peptide.
494 *Nucleic Acids Res*. **45**(16), 9788-9796 (2017).
495 33. Eric M. Scholar, William B. Pratt. *The Antimicrobial Drugs*. Oxford University Press.
496 34. Asher Brauner, Noam Shoresh, Ofer Fridman, Nathalie Q. Balaban. An Experimental
497 Framework for Quantifying Bacterial Tolerance. *Biophysical Journal*. **112**, 2664-2671
498 (2017).
499 35. Irit Levin-Reisman et al. Automated Imaging with ScanLag Reveals Previously
500 Undetectable Bacterial Growth Phenotypes. *Nature Methods*. **7**, 737-739 (2010).
501 36. Irit Levin-Reisman, Ofer Fridman, Nathalie Q Balaban. Scanlag: High-Throughput
502 Quantification of Colony Growth and Lag Time. *J. Vis. Exp*. **89**, 51456 (2014).

503 37. S E Luria, M Delbrück. Mutations of Bacteria from Virus Sensitivity to Virus Resistance.
504 *Genetics*. **6**, 491-511 (1943).
505 38. JoVe Science Education Database. Basic Methods in Cellular and Molecular Biology.
506 Bacterial Transformation: The Heat Shock Method. *JoVE*, Cambridge, MA, (2021).
507 39. Kirill A. Datsenko, Barry L. Wanner. One-Step Inactivation of Chromosomal Genes in
508 *Escherichia Coli* K-12 Using PCR Products. *Proc Natl Acad Sci*. **97**, 6640-6645 (2000).
509 40. Tomoya Baba et al. Construction of *Escherichia Coli* K-12 In-Frame, Single-Gene
510 Knockout Mutants: The Keio Collection. *Mol Syst Biol*, **2**, 2006.0008 (2006).
511 41. Hak Suk Chung et al. Rapid β-lactam-induced lysis requires successful assembly of the cell
512 division machinery. *PNAS*. **106**, 21872-21877 (2009).
513 42. Qian Peter Su et al. Superresolution Imaging Reveals Spatiotemporal Propagation of
514 Human Replication Foci Mediated By CTCF-Organized Chromatin Structures. *Proceedings*
515 *of the National Academy of Sciences*. **117**, 15036-15046 (2020).
516 43. Guangxi Wang et al., PTEN Regulates RPA1 and Protects DNA Replication Forks. *Cell*
517 *Res*. **25**, 1189-1204 (2015).
518 44. Bo Huang, Wenqin Wang, Mark Bates, Xiaowei Zhuang, Three-Dimensional Super-
519 Resolution Imaging by Stochastic Optical Reconstruction Microscopy. *Science*. **319**, 810-
520 813 (2008).
521 45. Florian Levet et al., SR-tesseler: A Method to Segment and Quantify Localization-Based
522 Super-Resolution Microscopy Data. *Nat. Methods*. **12**, 1065–1071 (2015).
523

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529

530 **Author contributions**

531 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.,
532 and D.J. wrote the manuscript with input from all co-authors. L.Z., G.P., Q.S., and Y.Y.C.
533 conducted experiments and analysed data.
534

535 **Competing interests**

536 Authors declare that they have no competing interests.

Data and materials availability

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

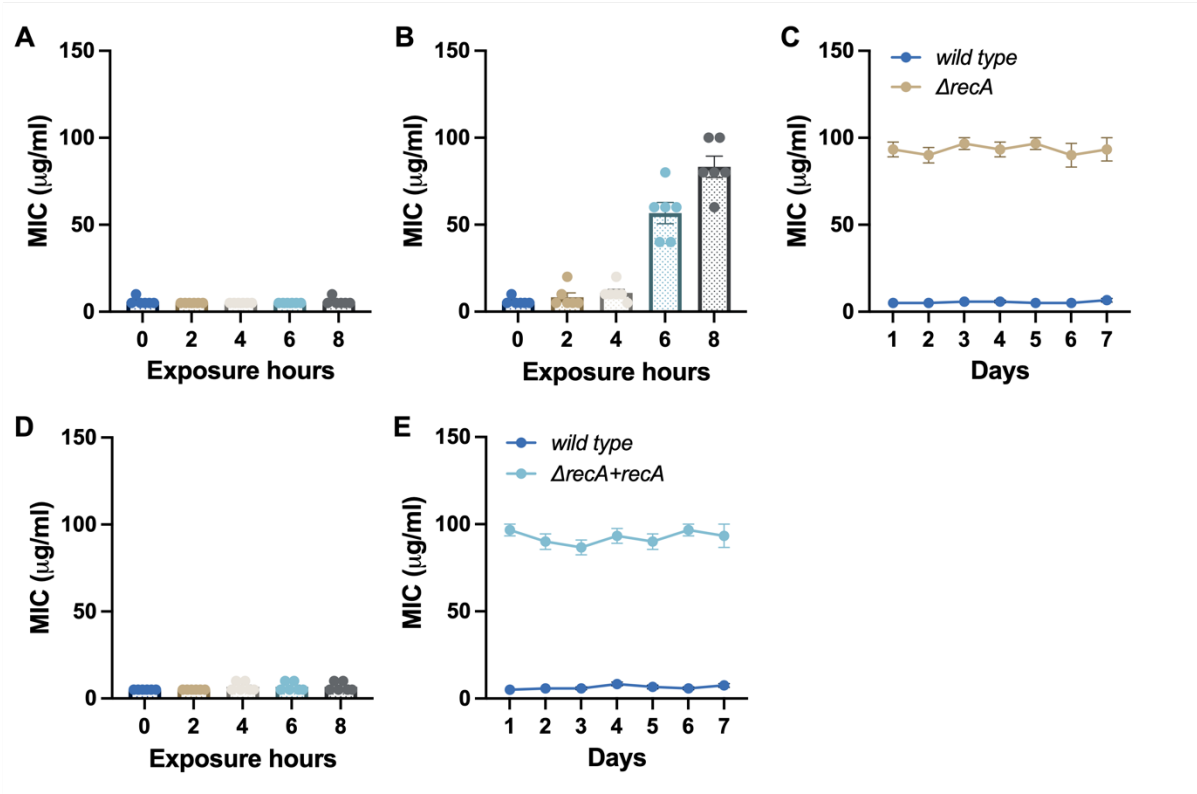


Figure 1. Superfast evolution of β-lactam resistance. The changes of MICs in the wild type (A) and the *ΔrecA E. coli* strain (B) after a single treatment with ampicillin at 50 μg/mL for 0, 2, 4, 6, and 8 hours. (C) After a single exposure to ampicillin at 50 μg/mL for 8 hours, surviving cells were continuously cultured in an antibiotic-free medium for 7 days, and MICs were measured at each day. (D) The changes of MICs in the *ΔrecA* strain, whose expression of RecA was restored before the single treatment with ampicillin at 50 μg/mL for 0, 2, 4, 6, and 8 hours. (E) After a single exposure to ampicillin at 50 μg/mL for 8 hours, the expression of RecA was restored in surviving cells, and cells were continuously cultured in an antibiotic-free medium for 7 days. MICs were measured at each day. Each experiment was independently repeated at least 6 times.

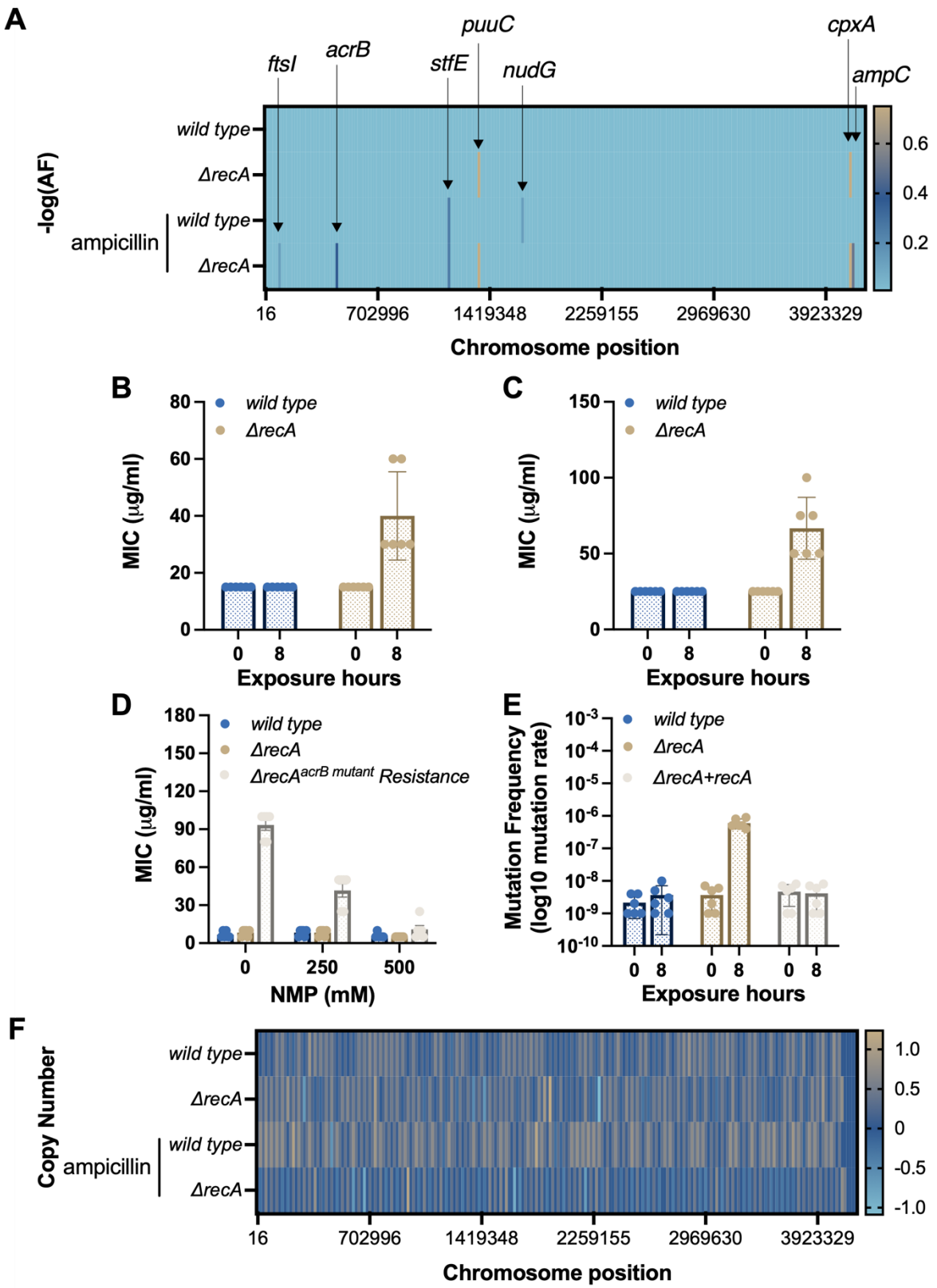
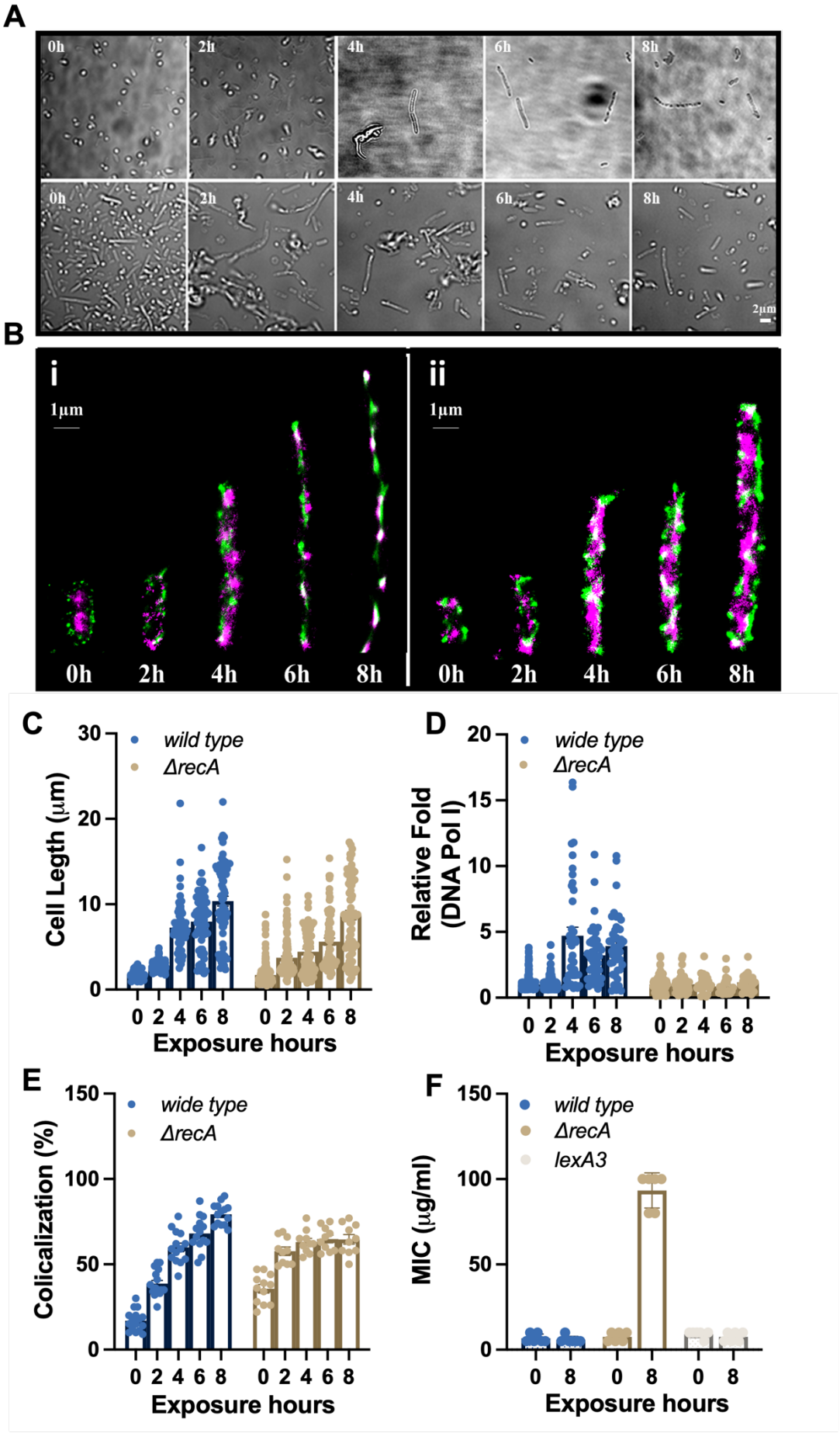


Figure 2. The single exposure to ampicillin caused the evolution of multi-drug resistance. (A) DNA mutations detected in the wild type and the *ΔrecA* strain before and after the single treatment with ampicillin at 50 μg/mL for 8 hours. After the exposure to ampicillin at 50 μg/mL

555 for 8 hours, surviving cells were tested for their resistance to (B) chloramphenicol and (C)
556 kanamycin. (D) The wild type, *ΔrecA*, and *ΔrecA* resistant isolates were treated with NMP at
557 0, 250, and 500 mM for 12 hours, and MICs were measured. (E) The changes of mutation
558 frequency in the wild type, *ΔrecA*, and *ΔrecA* strain whose expression of RecA was restored
559 before and after the single treatment with ampicillin at 50 μg/mL for 8 hours. (F) Before and
560 after the exposure of ampicillin at 50 μg/mL for 8 hours, the copy number variations of genes in
561 chromosome were measured in the wild type and *ΔrecA* strain. Each experiment was
562 independently repeated at least 4 times.


A
0h
2h
4h
6h
8h
2μm
B
i
ii
1μm
C
wild type
ΔrecA
Cell Legth (μm)
Exposure hours
D
wide type
ΔrecA
Relative Fold (DNA Pol I)
Exposure hours
E
wide type
ΔrecA
Colicalization (%)
Exposure hours
F
wild type
ΔrecA
lexA3
MIC (μg/ml)
Exposure hours

563

564 **Figure 3. The SOS-independent superfast evolution of antibiotic resistance.** (A) After the
565 single exposure of ampicillin at 50 μg/mL for 0, 2, 4, 6, and 8 hours, surviving cells were fixed
566 and imaged. Top, the wild type strain. Bottom, the *ΔrecA* strain. (B) Multinucleated filaments
567 were observed either in (i) the wild type or (ii) the *ΔrecA* strain after the single treatment with
568 ampicillin at 50 μg/mL for 0, 2, 4, 6, and 8 hours. Purple, *E. coli* chromosome. Green, DNA
569 polymerase I. (C) The length of filaments counted in the wild type and the *ΔrecA* strain. (D)
570 The relative fold of DNA polymerase I was measured in the wild type and *ΔrecA* strain. (E)
571 The co-localization between the chromosome and DNA polymerase I was calculated in the
572 wild type and *ΔrecA* strain. (F) After the single exposure to ampicillin at 50 μg/mL for 8 hours,
573 the MICs were measured in the wild type, *ΔrecA*, *lexA3* strain. Each experiment was
574 independently repeated at least 6 times.

575 **Table 1. DNA mutations detected in the wild type tolerant isolates and *ΔrecA* resistant**
576 **isolates.**

Gene	Mutation	Genomic Position	Phenotype
nudG	G > C	2398286	*wild type*^{Tolerance}
ftsI	A > C	93006	*ΔrecA*^{Resistance}
acrB	A > G	454273	
acrB	C > T	482387	
acrB	G > A	482581	
acrB	G > T	483413	
stfE	A>AGGTTTTCGAGAGC	1209618	
puuC	T > G	1363695	
cpxA	T > C	4104711	
ampC	G > A	4379014	
ampC	A > G	4379035	

577

Supplementary Information

For

Bacterial SOS-independent Superfast Evolution of Multi-drug Resistance

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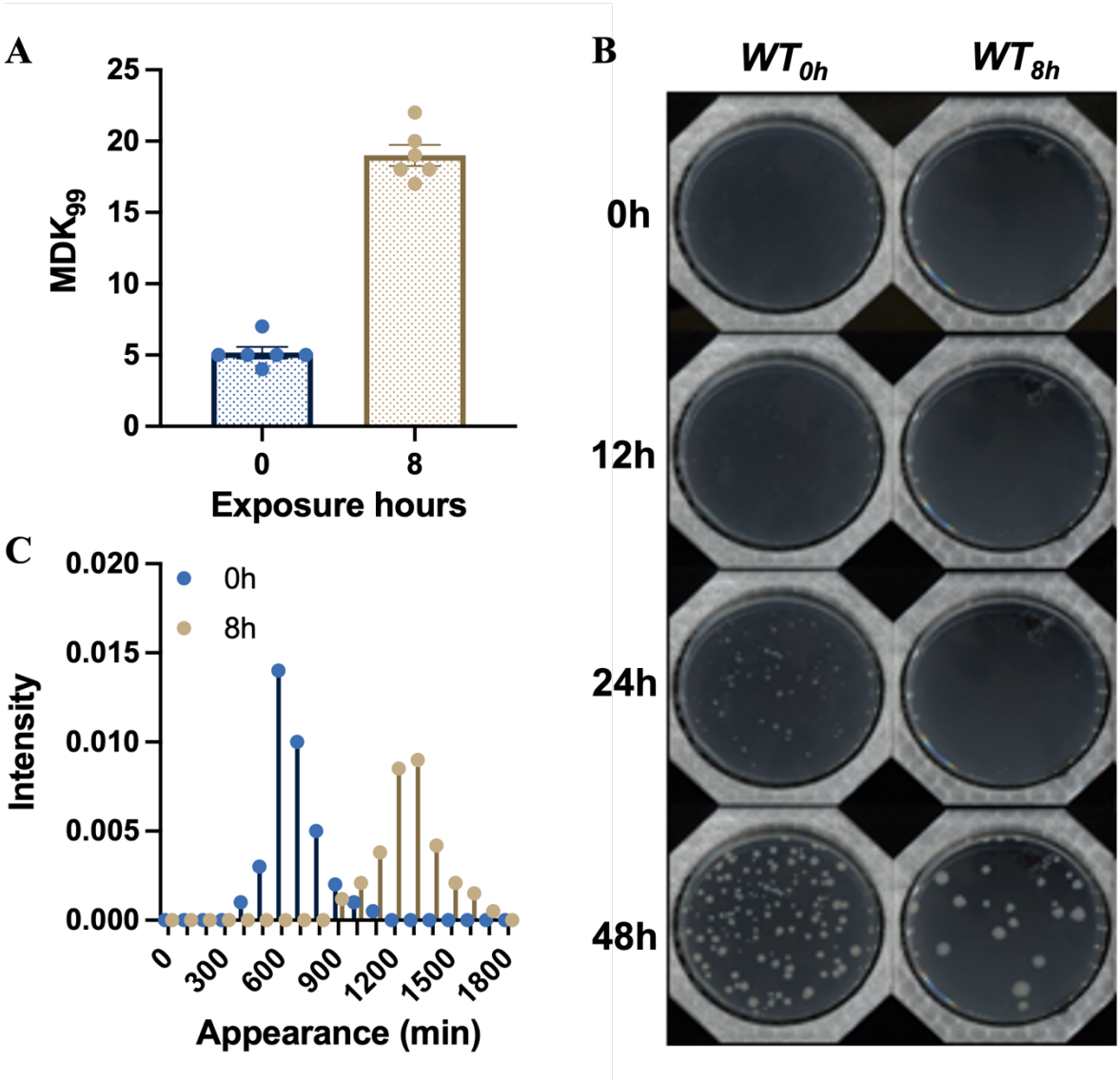


Figure S1. Emergence of tolerance in wild type strain after a single exposure to ampicillin. (A) Changes of the MDK_{99} in wild type strain after the exposure to ampicillin at 50 μg/mL for 8 hours. (B) The type of tolerance was defined by using the ScanLag system. The appearance and morphology of each single colony were automatically monitored. (C) Cells had an approx. 7 hours delayed appearance time showing an emergence of tolerance by lag-phase. Each experiment was independently repeated 6 times.

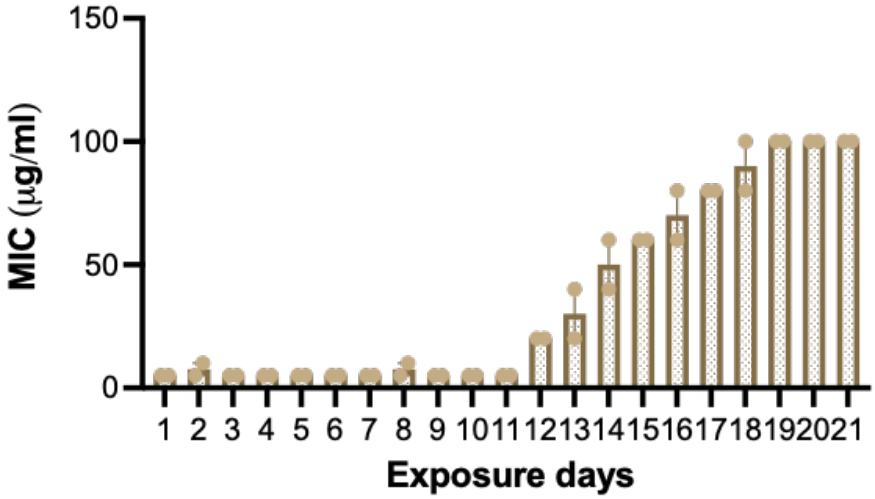


Figure S2. Evolution of resistance in the wild type strain. Changes of MICs in the wild type strain after 21 days treatment with ampicillin at 50 μg/mL for 4 hours at each day. MICs were measure at each day.

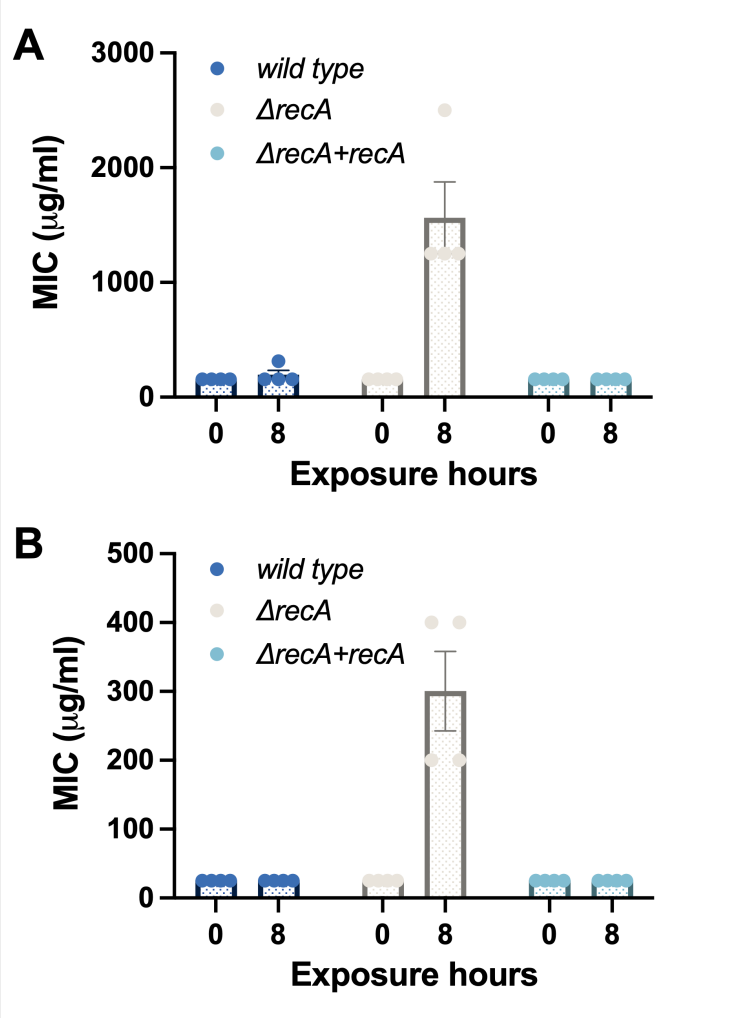
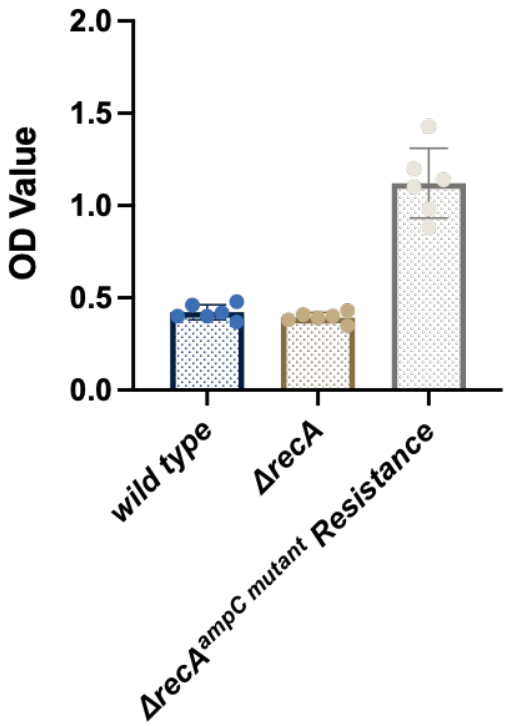


Figure S3. Evolution of antibiotic resistance in the *ΔrecA* strain. Changes of MICs in the *ΔrecA* strain treated with (A) penicillin G at 1 mg/mL, and (B) carbenicillin at 200 μg/mL for 8 hours. Each experiment was independently repeated 4 times.



33

34 **Figure S4. The level of β-lactamase was increased in the *ΔrecA* strain.** The *ΔrecA* strain
35 was treated with ampicillin at 50 μg/mL for 8 hours, and surviving isolates harbouring the
36 *ampC* mutations were selected. The level of β-lactamase in each strain determined by the
37 absorbance at OD500. The levels of β-lactamase in the wild type *ΔrecA* strain without the
38 exposure to ampicillin were tested as control. Each experiment was independently repeated 4
39 times.

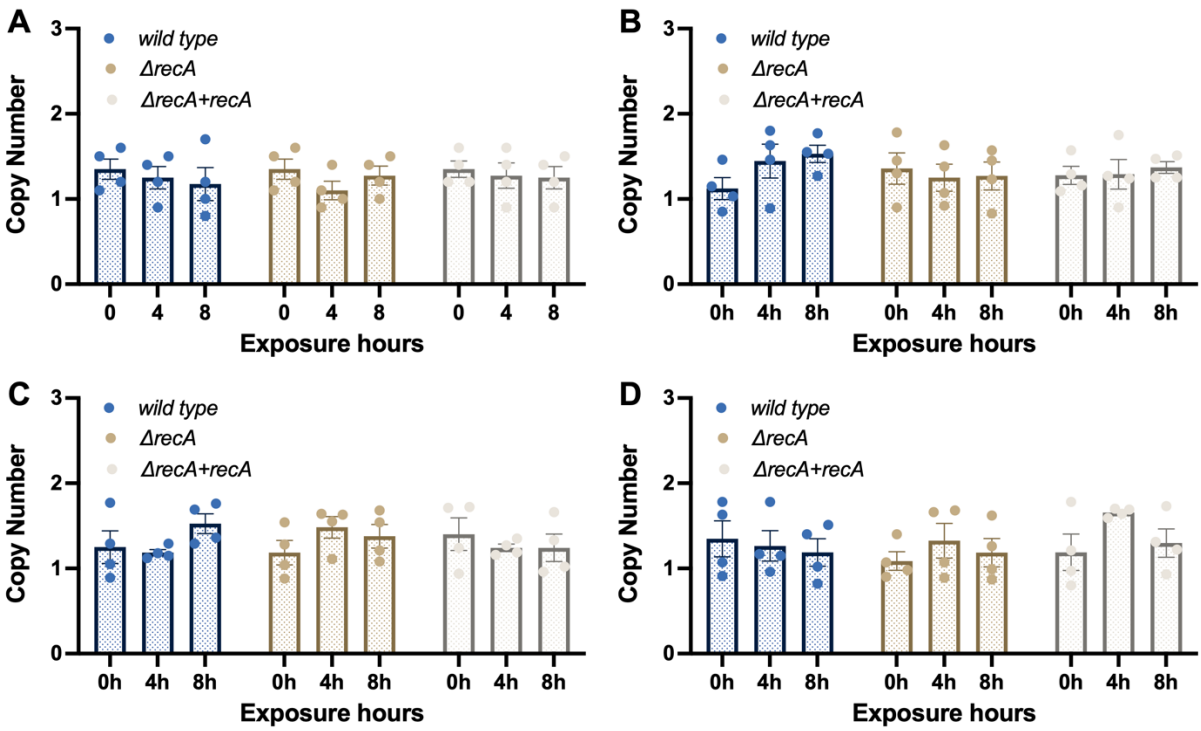


Figure S5. Copy number variations were not changed after the single exposure to ampicillin. After the treatment with ampicillin at 50 μg/mL for 0, 4, and 8 hours in each strain, copy number variations of the β-lactam-specific resistant genes were verified using the ddPCR method, including the gene (A) *ampC*, (B) *ampD*, (C) *ampE*, and (D) *ampG*. Each experiment was independently repeated 4 times.

47 **Table S1. DNA mutations detected in the wild type resistant isolates.**

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

48

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

Strain	Relevant Genotype	Parent strain	Source
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
RW1570	*recA*$^{+}$ *lexA3*	K-12	Lab stock

50

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

Plasmid	Relevant Genotype	Parent strain
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

52

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

Name	Sequence
recA-FWD	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAAC AAGACGAGAAACGAGAGAGGATGCTCAC
recA-REV	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGA GTAAAAGACGTCTAAGAAACCATTATTATCATGAC
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

54