PinR as a Stress Inducible Inhibitory Transcription Factor in Stopping Stress-induced Mutagenesis Bacterial Evolution of Antibiotic Resistance

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PinR is a lambdoid prophage Rac existing in many different bacteria, but its function is yet to be discovered. Here, we report PinR as an effective "anti-evolution" target towards combating antibiotic resistant bacteria. We show the function of PinR as a stress inducible inhibitory transcription factor in the bacterial evolution of antibiotic resistance, which represses the transcriptions of many oxidative stress response genes and consequently orchestrate the ROS burst-induced evolution of antibiotic resistance. We show that deletion of *pinR* gene can avoid the accelerated evolution of antibiotic resistance induced by the deficiency of DNA repair, while the strategies to target RecA, e.g., the deletion of *recA* gene, can be safely implemented to disarm the bacterial resistances to a variety of antibiotics.

Since the first antibiotic penicillin was discovered in 1940s (1), antibiotics are used to prevent and treat the bacterial infections. Antibiotics have been designed to target the cell wall or membranes, the bacterial DNA or RNA, and the machinery that produces proteins (2). However, the antibiotic resistance can occur naturally along with the administration of antibiotics, and the misuse of antibiotics is accelerating this process which poses a potentially catastrophic threat to the public health globally (3, 4).

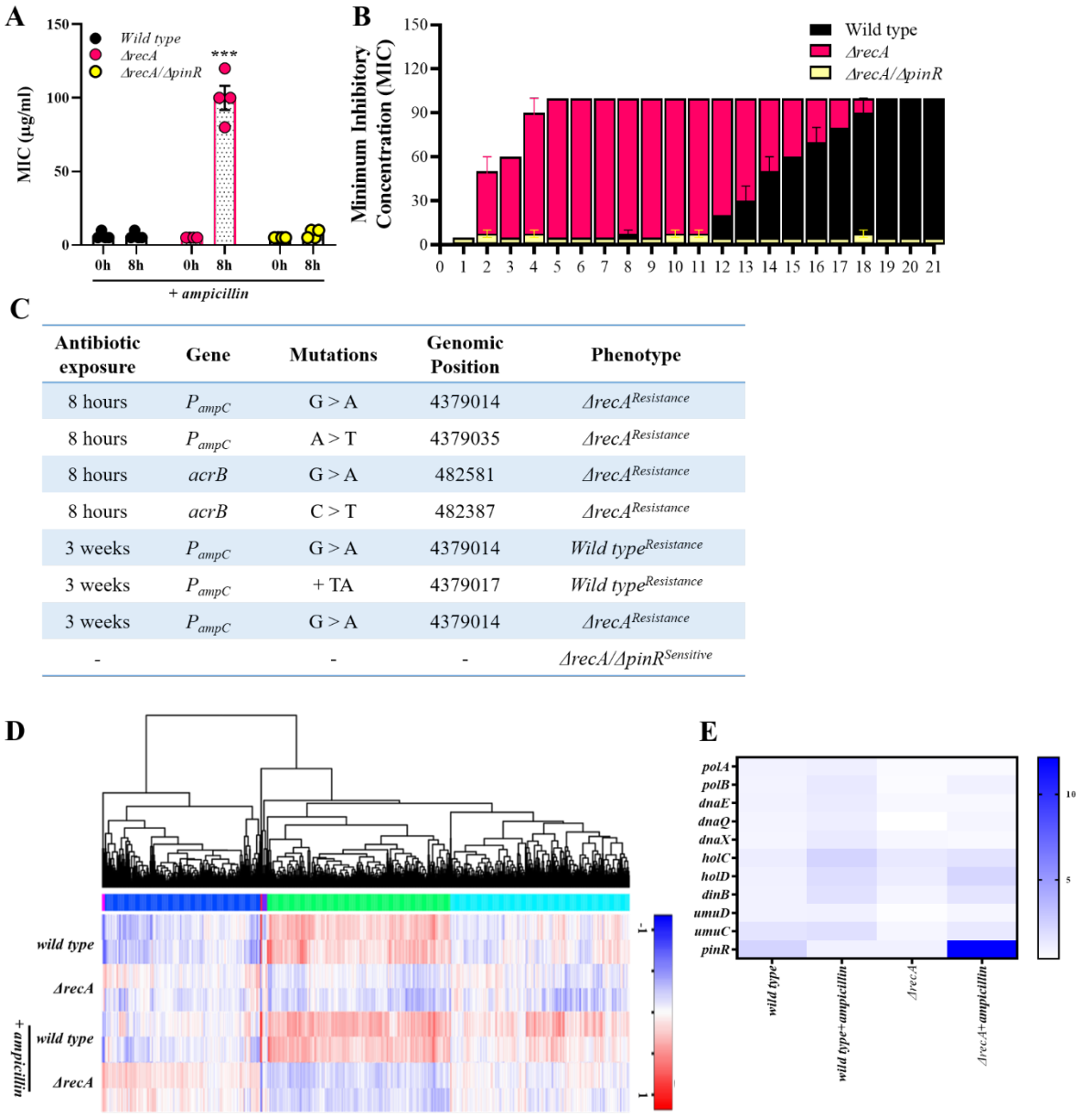
Bacterial antibiotic resistance can arise by either *de novo* mutations or bacterial recombination, in which the DNA recombinases catalyse site-specific recombination events within DNA and

35 promote the spread of antibiotic resistant genes (3, 4). The putative site-specific recombinase
36 PinR is a lambdoid prophage Rac encoded by the gene *pinR* (or named *ynaD*) and exists in
37 many bacterium types, including the *Escherichia coli* (*E. coli*), *Tetragenococcus halophilus,*
38 *Lactococcus lactis, Salmonella enterica and Streptococcus* (5-8). Although the function of
39 PinR is yet to be clear, it has been shown that PinR shares a similar structure with the DNA
40 invertase (Fig. S1) and can catalyse an inversion of a 177-bp DNA fragment in the
41 *Streptococcus* acting as a bacterial recombinase (6). Meanwhile, using MobileElementFinder,
42 the *pinR* is predicted as a mobile genetic element carrying the antibiotic resistant genes (7),
43 which has been experimentally evidenced in *L. pentosus* plasmid (8).
44
45 The SOS response pathway, involving its master regulator RecA, induces the DNA repair,
46 which consequently helps to drive the development and spreading of antibiotic resistance
47 determinants (9-11). Therefore, deactivating the RecA may disarm the bacterial resistance to
48 antibiotics (12). However, as a double-edged sword, the deficiency of DNA repair, as a result
49 of *recA* deletion, may also increase the rate of drug resistant mutagenesis induced by the
50 antibiotic exposure towards the antibiotic resistance (13, 14). Notably, our recent work reports
51 that the deletion of RecA can cause an unexpected superfast establishment of antibiotic
52 resistance in *E. coli ΔrecA* strain (15), but its mechanism is unknown.
53
54 Here, we find that, in the *ΔrecA* strain, further deletion of *pinR* not only prevented the
55 emergence of resistance after a single exposure to ampicillin (Fig. 1A), but also up to 3-week
56 intermittent treatment of ampicillin was unable to induce the evolution of resistance (Fig. 1B).
57 This was in a clear contrast to the commonly occurred resistance in the wild type strain treated
58 with an experimental evolution model that mimics the administration of antibiotics in clinic.
59 This suggests that targeting PinR offers a way out of the dilemma whether or not to target RecA
60 to suppress the SOS response, towards preventing the antibiotic resistance. As the deletion of
61 *recA* alone causes the DNA repair deficiency and consequently increases the rate of antibiotic
62 exposure-induced resistant mutagenesis that facilitates the antibiotic resistance, we further
63 sequenced the surviving isolates in *ΔrecA*/*ΔpinR* strain and confirmed that the drug resistant-
64 associated gene mutations were not detected in the *ΔrecA*/*ΔpinR* strain after neither the single
65 exposure to ampicillin for 8 hours nor the 3-week intermittent treatment with ampicillin (Fig.
66 1C). This further suggests the strategies to target both RecA and PinR may stop the bacteria
67 from developing the long-term antibiotic resistance.
68

69 To further understand the role of *pinR* and how the deletion of *pinR* can prevent the *ΔrecA*
70 strain from antibiotic resistance, we first compared the global transcriptome analysis in the
71 wild type and the *ΔrecA* surviving isolates after the single exposure to ampicillin. The treatment
72 of ampicillin affected the transcriptomic profile of both in the wild type and the *ΔrecA* strain,
73 compared with that of untreated control cells, with changes to the expression of 4373 and 4286
74 coding sequences, respectively (Fig. 1D). Venn diagram show the differentially expressed
75 genes with a $\log_2$ fold change ($\log_2$FC) of $\geq \pm 2$, and principal component analysis (PCA)
76 confirmed that the effect of ampicillin exposure on the *ΔrecA* strain remarkably differed from
77 that of the wild type cells (Fig. S2). Genome-wide expression changes were visualized as
78 volcano plots (Fig. S3) to identify specific genes with large fold changes and statistical
79 significance. With Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes
80 and Genomes (KEGG) pathway analysis shown in Fig. S4. We compared the selected
81 functional groups corresponding to biological processes, including DNA recombination,
82 quorum-sensing system, SOS response and ABC transport system (Fig. 1E and Fig. S5), and
83 found it is of much significance that a series of genes associated with the DNA damage and
84 repair were slightly changed, but the transcription of the gene *pinR* was considerably
85 upregulated (10-fold) in the *ΔrecA* resistant populations compared with that of the ampicillin-
86 treated wild type strain (Fig. 1E). These results collectively reveal that the PinR is a stress
87 inducible protein, and therefore as a new "evolution potentiator" of the antibiotic resistance
88
89 We further studied the oxidative stress response, which was uniquely regulated in the *ΔrecA*
90 strain and may cause the evolution of antibiotic resistance, as the induction of mutagenesis can
91 be stimulated by the production of reactive oxygen species (ROS) throughout the
92 administration of antibiotics in a few weeks (16). The transcriptome analysis revealed that the
93 single treatment of the *ΔrecA* strain with the ampicillin caused a significant downregulation of
94 transcription of many oxidative stress response genes, including the genes *cysJ, cysI, cysH,*
95 *soda* and *sufD* (Fig. 2A), indicating the upregulation of ROS after the single exposure to
96 ampicillin in the *ΔrecA* strain. By adding 50 mM glutathione (GSH), a natural antioxidant, into
97 the *ΔrecA* culture medium and exposing them to 50μg/ml ampicillin for 8 hours, we observed
98 that the antioxidant prevented the emergence of resistance to ampicillin in the *ΔrecA* strain (Fig.
99 2B) but did not affect the survival rate (Fig. 2C), which suggested that the overproduction of
100 ROS was not a lethal effect induced by the single exposure to ampicillin in the *ΔrecA* strain.
101 We sequenced the survived isolates and found that the addition of GSH prevented the DNA
102 mutations associated with the drug resistance in the *ΔrecA* strain (Fig. 2D). These data

demonstrated that the ROS burst happened as the result of the suppression on the oxidative stress response system, and consequently triggered the resistant mutations, which caused the superfast emergence of resistance in the *ΔrecA* strain. More importantly, by further comparing the transcription of oxidoreductase genes in the *ΔrecA/ΔpinR* strain, we did not find the changes of the transcription of any oxidative stress response genes in the *ΔrecA/ΔpinR* strain after the single exposure to ampicillin (Fig. 2A). These results, taking together, indicated that the PinR services as an inhibitory transcription factor repressing on the transcription of oxidoreductase genes, and the absence of PinR stops the evolution of antibiotic resistance caused by the overaccumulation of ROS.

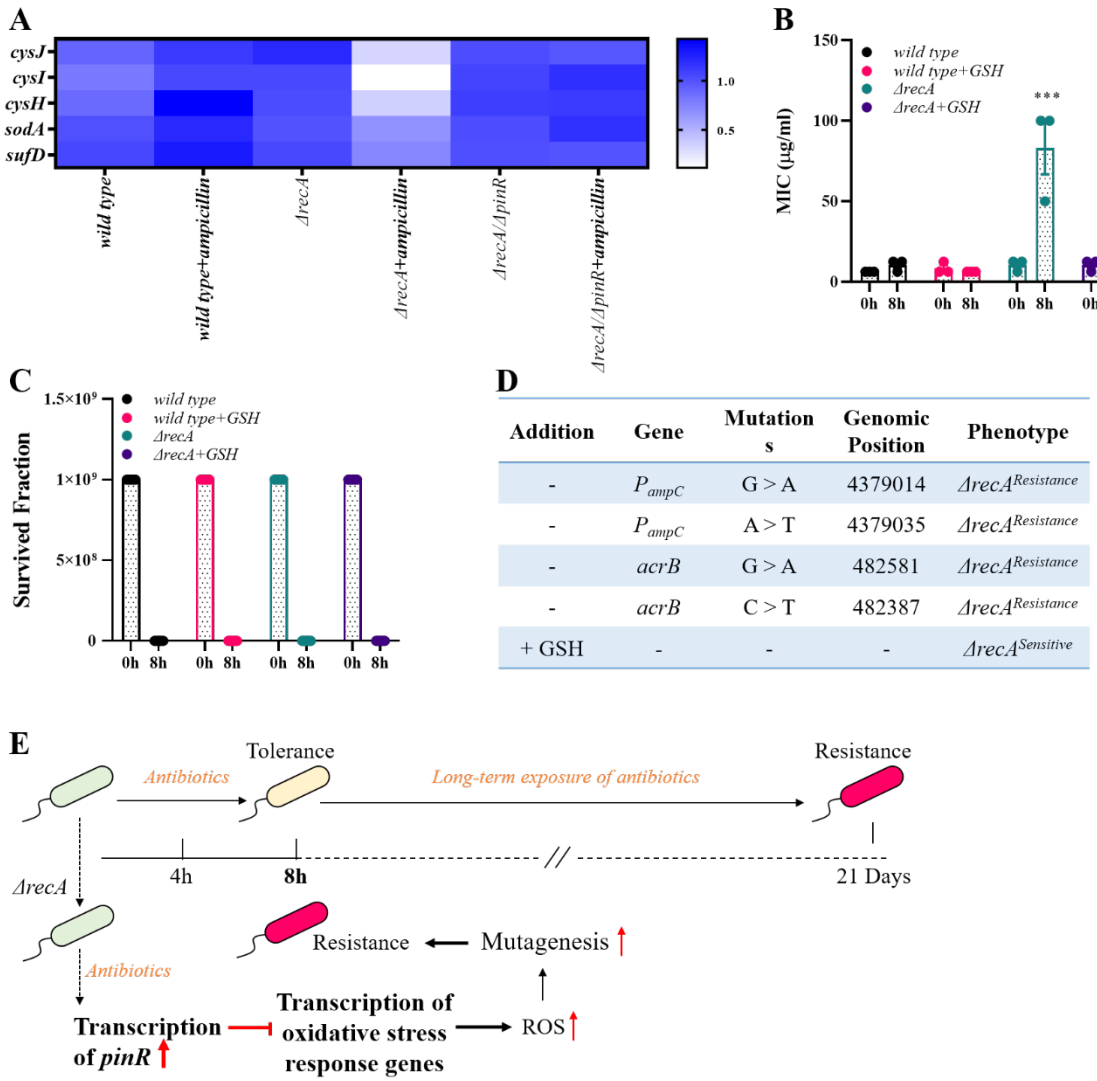
In short, we conclude PinR as an inhibitory transcription factor in the pathway indicated in Fig. 2E, as the transcription of *pinR* can be upregulated in the *ΔrecA* strain under the treatment of ampicillin. Although the involvement of ROS in the evolution of antibiotic resistance has been identified, we first find that the upregulation of PinR can orchestrate the ROS burst induced mutagenesis through the repression on the oxidative stress response system, which consequently facilitates the bacterial evolution of antibiotic resistance. From a drug development perspective, strategies to directly suppress the transcription of 'potentiator' genes or to inhibit their encoding proteins activities may combat the antibiotic resistance (8). Our result shows the deletion of *pinR* can dramatically delay the evolution of resistance to ampicillin in both the short-term and long-term models. With the role of PinR being clear, our evolutionary model can be generic, as the overproduction of ROS is a common action upon the treatment of many antibiotics (16), maintaining the transcription of oxidative stress response genes by inhibiting the transcription of 'potentiator' genes will become a direction to stop the stress-induced mutagenesis that is otherwise unavoidably induced by the treatments of a variety of antibiotic drugs. Therefore, our work suggests future studies on the 'potentiator' genes towards the safe and more effective strategies to be developed for infectious disease treatments.



Antibiotic exposure	Gene	Mutations	Genomic Position	Phenotype
8 hours	P_{ampC}	G > A	4379014	$\Delta recA^{Resistance}$
8 hours	P_{ampC}	A > T	4379035	$\Delta recA^{Resistance}$
8 hours	*acrB*	G > A	482581	$\Delta recA^{Resistance}$
8 hours	*acrB*	C > T	482387	$\Delta recA^{Resistance}$
3 weeks	P_{ampC}	G > A	4379014	$Wild\ type^{Resistance}$
3 weeks	P_{ampC}	+ TA	4379017	$Wild\ type^{Resistance}$
3 weeks	P_{ampC}	G > A	4379014	$\Delta recA^{Resistance}$
-		-	-	$\Delta recA/\Delta pinR^{Sensitive}$

Figure 1. PinR as a new "evolution potentiator" of the antibiotic resistance. (A) Deletion of *pinR* delayed the evolution of antibiotic resistance after an intermittent treatment with ampicillin (50 μg/ml) for 21 days in the *ΔrecA/ΔpinR* strain. **(B)** Deletion of *pinR* prevented the evolution of antibiotic resistance in the *ΔrecA/ΔpinR* strain after a single exposure to ampicillin at 50 μg/ml for 8 hours. **(C)** DNA mutations detected in the wild type, *ΔrecA*, and *ΔrecA/ΔpinR* strains after a single exposure to ampicillin (50 μg/ml) for 8 hours or an intermittent treatment of ampicillin at 50 μg/ml for 21 days. **(D)** The clustered heatmap of relative expression of coding sequences in the wild type and the *ΔrecA* strain. **(E)** After the exposure to ampicillin at 50 μg/ml for 8 hours, the regulation of transcription of DNA recombination and repair genes. The global transcriptome sequencing was performed twice.

Other experiments were independently repeated at least 4 times. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Addition	Gene	Mutations	Genomic Position	Phenotype
-	P_{ampC}	G > A	4379014	$\Delta recA^{Resistance}$
-	P_{ampC}	A > T	4379035	$\Delta recA^{Resistance}$
-	*acrB*	G > A	482581	$\Delta recA^{Resistance}$
-	*acrB*	C > T	482387	$\Delta recA^{Resistance}$
+ GSH	-	-	-	$\Delta recA^{Sensitive}$

Figure 2. PinR orchestrates the ROS burst induced mutagenesis facilitating the bacterial evolution of antibiotic resistance. (A) After the exposure to ampicillin at 50 μg/ml for 8 hours, the regulation of the transcription of oxidative stress response genes in the wild type, *ΔrecA*, and the *ΔrecA/ΔpinR* strain. **(B)** Addition of 50 mM antioxidative compound GSH prevented the evolution of antibiotic resistance in the *ΔrecA* strain. **(C)** Survival fraction after the exposure to ampicillin in the wild type and the *ΔrecA* strain with or without the addition of GSH. **(D)** DNA mutation detection after the single treatment of ampicillin (50 μg/ml) in the *ΔrecA* strain with or without the addition of GSH. **(E)** The mechanism by PinR mediating the bacterial evolution of antibiotic resistance. The global transcriptome sequencing was performed twice. Other experiments were independently repeated at least 4 times. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

157 **Materials and Methods**

158 **Bacterial strains, medium and antibiotics**

159 Bacterial strains and plasmids used in this work are described in Table S1 and Table S2. Luria-
160 Bertani (LB) was used as broth or in agar plates. *E. coli* cells were grown on LB agar (1.5%
161 w/v) plates at 37ºC, unless stated otherwise, antibiotics were supplemented, where appropriate.
162 Whenever possible, antibiotic stock solutions were prepared fresh before the use.

163

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

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

171

172 To test resistance, the surviving isolates were first resuspended in 30 ml LB medium and grown
173 overnight at 37ºC with shaking at 250 rpm. The regrown culture was then plated onto LB agar
174 supplemented with the appropriate selective antibiotics and incubated 16 hours at 37ºC. Single
175 colonies were isolated and used to test the resistance or stored at -80ºC for future use.

176

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

184

185 **Antibiotic susceptibility testing**

186 The susceptibility of *E. coli* cells to antibiotics was measured by using minimum inhibitory
187 concentration (MIC) testing (17).

188

189 **Construction of deletion mutants**

190 Lambda Red recombination was used to generate various gene deletions in *E. coli* strains
191 followed by previous reported methods with modifications (18, 19). Primers used in this work
192 are listed in the Table S3.
193
194 **DNA extraction**
195 Chromosomal DNA was extracted and purified using the PureLink™ Genomic DNA mini kit
196 (ThermoFisher Scientific). Plasmid DNA was extracted and purified using the PureLink™
197 Quick Plasmid Miniprep kit (ThermoFisher Scientific).
198
199 **Whole genome sequencing**
200 The genomic sequencing was conducted following the Nextera Flex library preparation kit
201 process (Illumina), and processed by Sangon Biotech, Shanghai, China.
202
203 **Global transcriptome sequencing**
204 The global transcriptome sequencing was processed and analysed by Genewiz, Jiangsu, China.
205 Primers used in this work are listed in the Table S3.
206
207 **Statistical analysis**
208 Statistical analysis was performed using GraphPad Prism v.9.0.0. All data are presented as
209 individual values and mean or mean ± s.e.m. A two-tailed unpaired Student's t-test using a 95%
210 confidence interval was used to evaluate the difference between two groups. For more than two
211 groups, a one-way ANOVA was used. A probability value of $P < 0.05$ was considered
212 significant. Statistical significance is indicated in each figure. All remaining experiments were
213 repeated independently at least fourth with similar results.
214

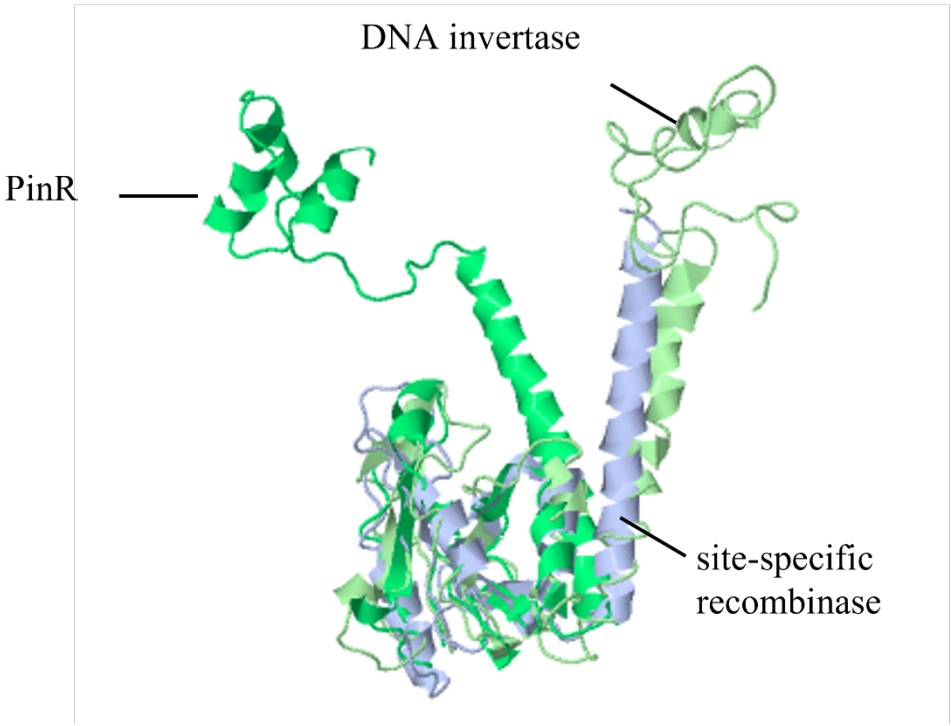


Figure S1. The proposed structure of PinR compared with the site-specific DNA recombinase and DNA invertase.

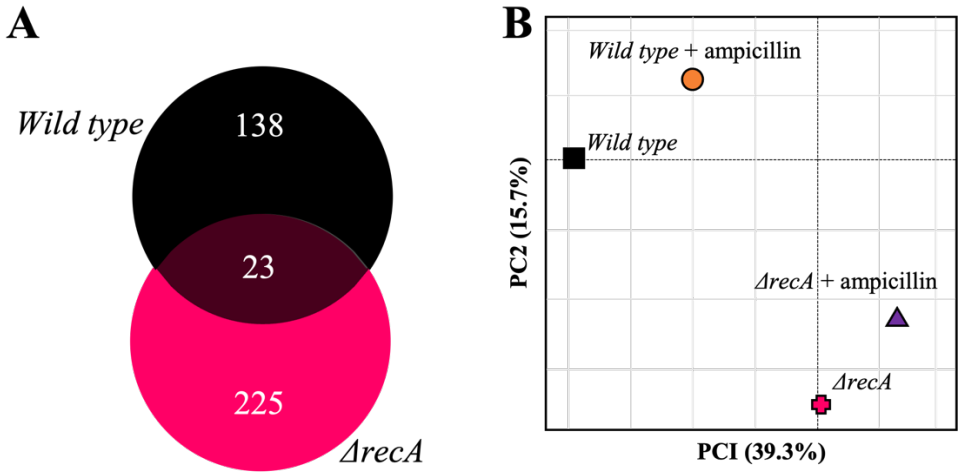


Figure S2. Transcriptional response of individual strain treated with ampicillin for 8 hours. (A) Venn diagram of differentially expressed genes ($\log_2 FC \geq \pm 2$). (B) Bi-plot of the principal-component analysis of normalized read counts for all strains.

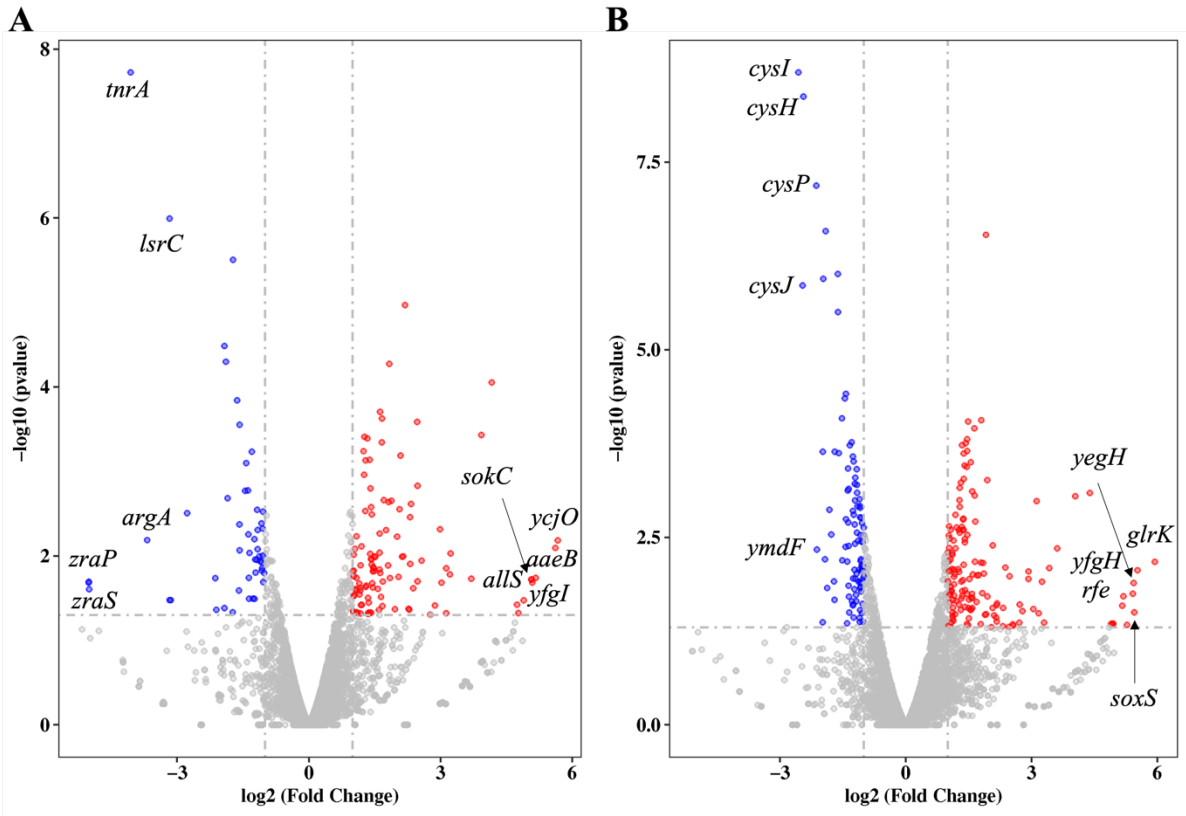


Figure S3. Summary of genome-wide expression changes in the wild type strain (A) and the *ΔrecA* strain (B) after the single exposure to ampicillin. The top 10 most differentially expressed genes are labelled in each plot. Gray dots indicate genes with no significant difference compared to the untreated control ($P > 0.05$), blue dots indicate genes with a significant difference compared to the untreated control ($P < 0.05$), and red dots indicate genes with both a significant difference ($P < 0.05$) and $\log_2$FC of > 2 compared to the untreated control, with numerical annotations to indicate the number of differentially expressed genes.

Figure S4. Transcriptional response of the wild type and *ΔrecA* strains after the exposure to ampicillin at 50 μg/ml for 8 hours. GO enrichment analysis of different genes including in biological process (red), cellular component (green), and molecular function (white) in the wild type strain (A) and the *ΔrecA* strain (B). KEGG pathway enrichment scatter plot of different genes in (C) the wild type strain and the *ΔrecA* strain (D). Top 20 enrichment pathways were listed in the GO and KEGG enrichment analysis.

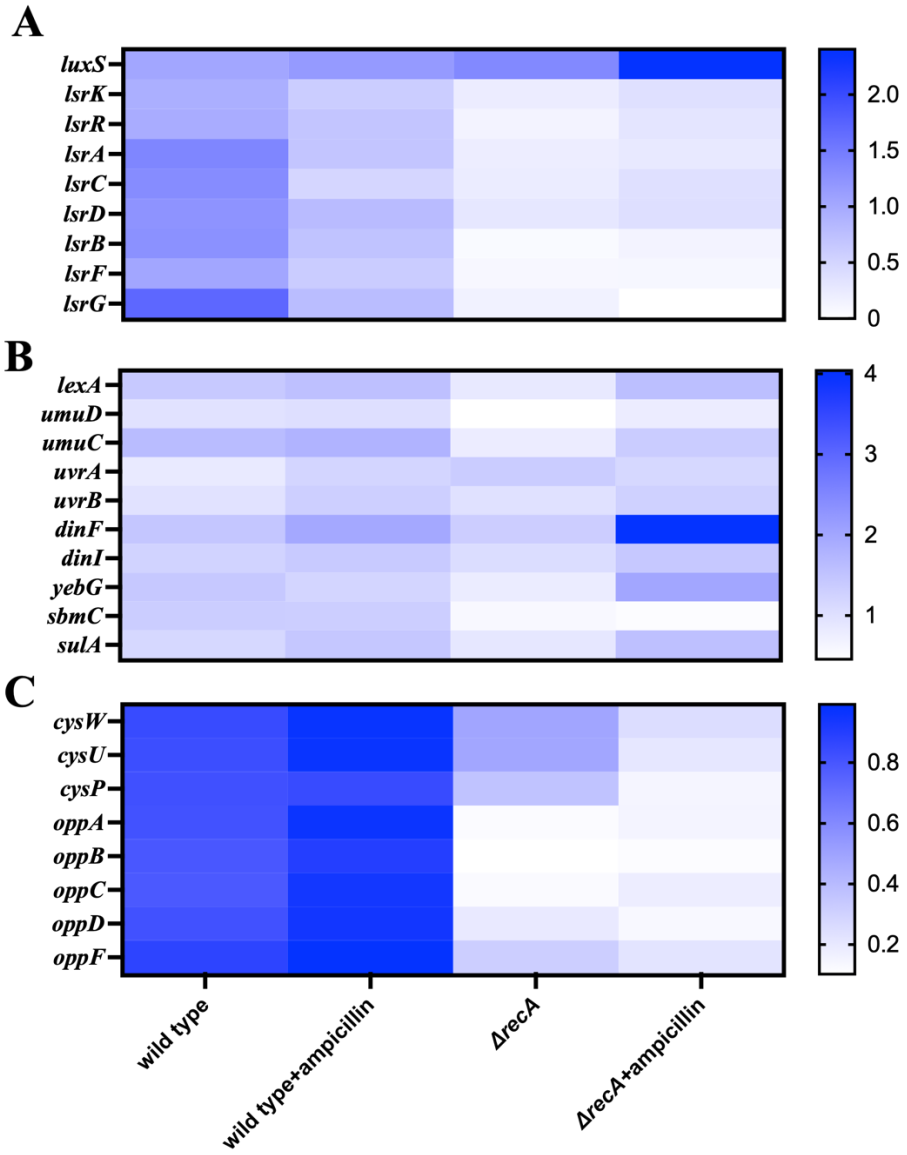


Figure S5. Heatmaps show $\log_2$FC data for each strain treated with single exposure to ampicillin at 50 μg/ml for 8 hours. (A) Quorum-sensing system genes. (B) SOS response genes. (C) ABC transport system genes.

Table S1. Strains used in this study.

Strain	Relevant Genotype	Parent strain	Source/technique
DH5α	-	-	Lab stock
E. coli K-12	*recA*$^+$ *pinR*$^+$	-	Lab stock
LZ101	*ΔrecA::Tet*	*K-12*	Lab stock
LZ102	*ΔrecA::Tet*, *ΔpinR::Kan*	LZ101	This study

249 **Table S2. Plasmids used in this study.**

Plasmid	Description	Source
pKD46	Helper plasmid for Lambda RED recombination	Lab stock

250

251 **Table S3. Primers used in this study.**

Name	Sequence
recA-FWD	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAAC AAGACGAGAAACGAGAGAGGATGCTCAC
recA-REV	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGA GTAAAAGACGTCTAAGAAACCATTATTATCATGAC
pinR-FWD	CAAGGAAGGGGGCTTGGAAGACGTAAAGCATCTCACACCGAGA TTATTTTCATATGAATATCCTCCTTAGTTCCTATTC
pinR-REV	TTTCTGAGATGCATTATGATATGAACACCAATTTCGTATAGAGTC TCACTGAGCTGCTTCGAAGTTCCTA
P5 adapter	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
P7 adapter	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

252

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