SUPERFAST EVOLUTION OF BACTERIAL RESISTANCE TO BETA-LACTAM ANTIBIOTICS MEDIATED BY BACTERIAL DNA RECOMBINATION

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Certificate of Original Authorship

I, Le Zhang declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the IBMD, Faculty of Science at the University of Technology Sydney. This thesis is wholly my own work unless otherwise reference or acknowledged. In addition, I certify that all information sources and literatures used are indicated in the thesis. This document has not been submitted for qualifications at any other academic institution.

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List of Publications

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- [1] Jin D*, Xi P*, Baoming Wang, **Zhang L**, Enderlein J, van Oijen AM. Nanoparticles for super-resolution microscopy and single-molecule tracking. Nature Method. 2018, 15: 415-423.

- [2] Tingting Chen, Anna B. Liu, Shili Sun, Nadim J. Ajami, Matthew C. Ross, Hong Wang, **Le Zhang**, Kenneth Reuhl, Koichi Kobayashi, Janet C. Onishi, Liping Zhao, Chung S. Yang*. Green Tea Polyphenols Modify the Gut Microbiome in db/db Mice as Co-Abundance Groups Correlating with the Blood Glucose Lowering Effect. Molecular nutrition & food research. 2019, 63: 1801064.

- [3] **Le Zhang**, Qian Peter Su, Zhichao Kang, Yuen Yee Cheng, Yan Liao, Nural Cokcetin, Amy L. Bottomley, Qiongfang Li, Iain Duggin, Andrew Robinson, Elizabeth J. Harry, Antoine v. Oijen, Dayong Jin*. Superfast bacterial evolution of resistance to β-lactam antibiotics mediated by bacterial DNA recombinases. Science. 2020. (Submitted).

Abstract

The emergence of antibiotic resistance is a global problem. Many studies show that sub-lethal concentration of antibiotic exposure or transit exposure to antibiotics can induce the formation of persistence or tolerance; whereas, cyclic exposure (usually 8-17 days) of antibiotics promotes the accumulation of bacterial adaptive mutations in persisters towards the subsequent evolution of resistance. However, the antibiotics induce mutagenesis that damage bacterial DNA has been poorly understood. Notably, the induction of SOS response has always been believed to aid bacterial propagation defense against antibiotic lethality, which requires the activation of SOS-promoting *recA*. A recent study implicated the essential role of RecA in the evolution of resistance to fluoroquinolones antibiotics, but it is unclear whether this is a conserved mechanism of resistance in response to different antibiotic classes.

RecA has been considered as a drug target to suppress the induction of SOS response towards the evolution of resistance caused by the broad-spectrum fluoroquinolone antibiotics. Here, we report that single exposure of β-lactam antibiotics can trigger a superfast evolution of resistance in *recA* deletion *E. coli* strain, independent to the SOS response. This type of single exposure causes gene mutations on an uncharacterized gene *pinR*, and its encoded protein may be involved in DNA recombination. Moreover, single or intermittent treatment of β-lactam antibiotics fails to induce the resistance in the *pinR* deletion *E. coli* strain. This work highlights the antagonistic role among DNA recombinases in the emergence of antibiotic resistance, and demonstrates that loss of *recA* increases the rate of resistance to β-lactam antibiotics, but PinR is likely to be a novel drug target.

Key words: Antibiotic tolerance, heritable resistance, RecA, *pinR*, *ampC*,

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Chapter 1. Introduction

1. Introduction

1.1 Bacterial persistence to antibiotics

Antibiotic resistant infections are presently a global threat to public health policy [1]. However, bacterial persistence is increasingly recognized as another major cause of antibiotic treatment failure and relapsing infections [2-4]. Unlike bacterial resistance to antibiotics that confers bacterial inheritable ability of being survival in the presence of antibiotics, bacterial persistence is identified based upon the formation of rare persisters cells that transiently display tolerance to antibiotic killing [5]. Typically, persister cells protect against antibiotic killing by switching to a physiologically dormant state, that is, bacterial cells reduce essential metabolism and stop growing. The presence of persister cells can be identified by the "biophasic killing" phenomenon of bacterial cultures when exposured to antibiotics: initially, antibiotics rapidly kill bacterial cells indicating the death of majority of population, followed by a secondary phase with much slower killing efficiency representing the formation of persister cells (Fig. 1.1) [6]. Antibiotic tolerance permits the growth of bacteria after the termination of antibiotic treatment, and has been identified as a major cause for the relapsing course of many bacterial infections [2, 3].

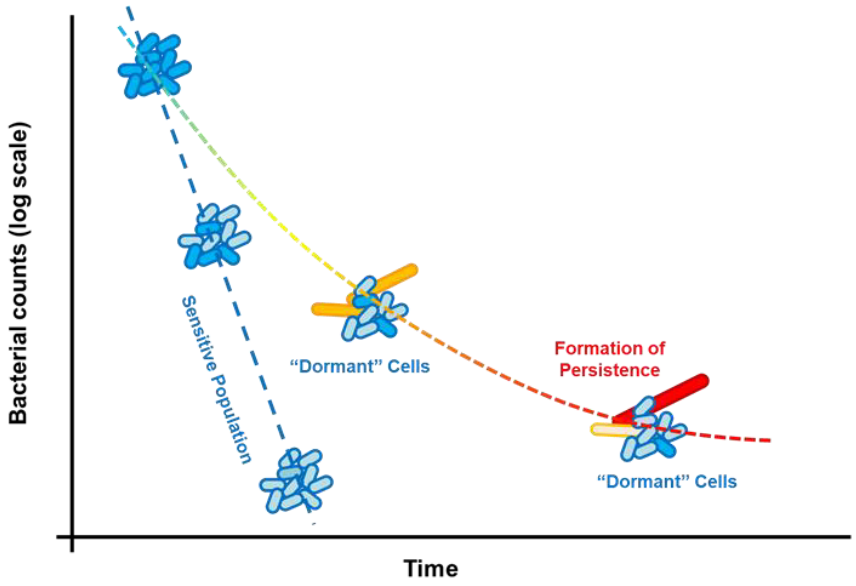

Figure 1.1 Biphasic killing kinetics of bactericidal antibiotic treatment. *A single lethal exposure of bacterial antibiotic can rapidly eradicate the sensitive population (blue) at initial exposure until the formation of non-growing persister cells or emergence of slow-growing persister cells (yellow) that are killed at a lower rate. The slower killing has been interpreted to reflect the persister resuscitation rate, but this remains to be substantiated experimentally. However, the termination of antibiotics treatment enables the population to be replenished by resuscitation of surviving persister cells.*

1.1.1 Clinical relevance of persister cells

Nowadays, increasing numbers of patients are suffering from persistent bacterial infections which defy massive, long-lasting and repeated antimicrobial treatment. It is known that these persistent infections are resulted from non-full clearance of bacterial

cells by antibiotics since bacterial cells can survive through the formation of biofilms or other protected niches [7, 8]. It includes urinary tract infections with uropathogenic strains of *Escherichia coli*; the notoriously recalcitrant infections with *Mycobacterium tuberculosis*, or opportunistic infections of implanted devices, open wounds, and other body lesions typically caused by biofilms of *Pseudomonas aeruginosa* or *Staphylococcus aureus* [7, 9].

It has been observed that heterogeneous populations of persister cells exist in many models of bacterial infections which display antibiotic tolerance, slow growth, and the ability to reinitiate infection after antibiotic treatment [9-14]. The standard protocol to treat chronic infections is based on a cyclic administration of high dose of antibiotics, but this treatment is clearly associated with greatly increased level of persister cells and repeatedly shown to select for resistant mutants [15-23].

1.1.2 Dormancy and the physiology of persister cells

Antibiotics kill bacterial cells through the damage of various cellular processes [24]. Whereas, the survival of persister cells is usually explained by a transition into a certain state typically called “dormant”, in which bacteria reduces the growth rate and metabolism to protect the cellular processes otherwise poisoned by bacterial antibiotics [25, 26]. In 1944, Joseph Bigger was first to report that after antibiotics treatment some

cells survived in a “dormant, non-dividing phase” [27]. Previous finding reported that using flow cytometry most of the dormant cells are not antibiotics tolerant, even though larger amounts of persister cells can be found in the physiologically dormant fraction of the population [28-30]. Therefore, it has been evident that persister cells are not simply dormant cells, their formation is supposed to be involved with specific physiological changes.

1.1.3 Persister cell formation in bacterial populations

Many experiments demonstrate that the formation of persister cells is typically controlled by a cellular signalling induction, and its mechanisms are directly regulated by genetic encoding [31-35]. Meanwhile, genetic heritability enables bacterial cells higher formation frequency of being persisters which further make persisters adapt to the incidence of antibiotics treatment. For example, cyclic exposure of antibiotics has been evidently linked with the increased levels of persisters in clinical isolates [36]. However, even though the evolution of bacterial persistence is rather low, the formation of persister cells also has a pleiotropic fitness cost which can reduce the formation of persister cells in the absence of antibiotics. In natural populations, the forces driving the evolution of bacterial persistence are variable since persister levels among different species and strains are different [37-39].

It has been well-known that the formation of bacterial persister cells is driven by a combination effect between stochastic and responsive mechanisms which regulates an induction of bacterial responses to increased environmental stress when harmful conditions are present [40]. Persister cells can be formed before the exposure of lethal dosage of antibiotics. Direct observations from single cell microfluidics and flow cytometry show that dormant cells pre-exist in the subpopulation of exponentially growing *E. coli* before the antibiotic treatment and survive after the exposure [41-42]. Furthermore, bacterial cells can respond to environmental changes via quantitatively and qualitatively modulating the rate of phenotypic conversion into persister cells [43]. For example, sublethal concentration of antibiotics can stimulate the formation of bacterial persister cells both *in vivo* and *in vitro* [44, 45].

1.1.4 Cellular signalling upstream of persister formation

Both stochastic and responsive persister cells formation are controlled by various signalling pathways, which includes stringent response and (p)ppGpp signalling, RpoS responses, and bacterial communication (Fig. 1.2).

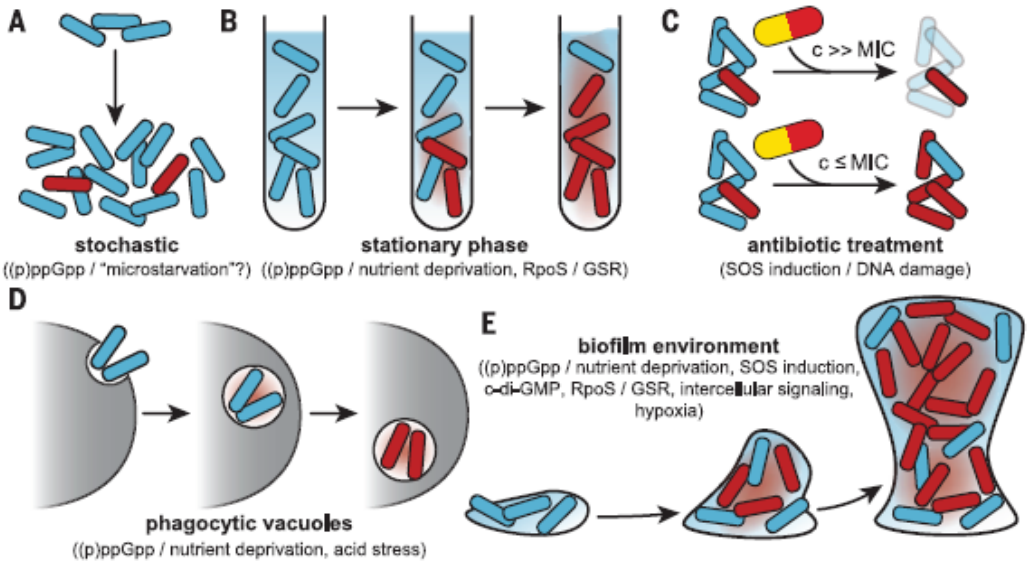

Figure 1.2 Environmental cues and cellular signals underlying persister cells formation [46]. *(A) Bacterial cells can be stochastically induced to be persister cells. (B) Changes of environmental stress can induce bacterial evolution of persistence which is strongly induced by the stationary phase. (C) sublethal antibiotic treatment, and (D) phagocytosis by immune cells, as well as (E) in biofilm.*

Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) are two ubiquitous second messengers which are collectively involved in the bacterial stringent response that is typically produced as a result of increased environmental stress in order to reprogram cellular physiology from growth to metabolic homeostasis and survival functions [47, 48]. In bacterial cells, (p)ppGpp is regulated by a synthetase RelA which can be activated by amino acid starvation or heat shock. (p)ppGpp modulates many bacterial physiology process by transcriptional reprogramming and the direct adjustment

of target protein activities [47-49]. Whereas, mutants of RecA fail to generate (p)ppGpp which further reduce the formation of persister cells. For example, it is observed that *E. coli* cells with relA mutants are hardly triggered to be persister cells during experimental growth, in biofilms and in the stationary phase [50, 51].

In addition, the formation of bacterial persister cells is able to be induced by a stochastic activation of (p)ppGpp during exponential growth and by certain environmental condition in which the production of (p)ppGpp is stimulated, such as in biofilms or during the stationary phase (Fig. 1.2). Bacterial biofilms are surface-associated multicellular communities typically harbouring 100-1000 fold more persister cells than planktonic cultures [52, 53], which relies on the activation of (p)ppGpp and other signalling messengers, including hypoxia, sessility-associated second messengers, or SOS induction [53-55].

1.2 Molecular mechanisms of bacterial resistance to antibiotics

Since the first antibiotic penicillin was found at 1928, antibiotics have reduced childhood mortality, increased our life expectancy and even been crucial for invasive surgery and treatments [56-57]. However, a realistic and increasing global fact is that antibiotic resistance has been one of the greatest threats to human health. World Economic Forum Global Risks reports that, in Europe, around 25,000 people die each year resulted from the infection by multidrug-resistant bacteria, and it costs the European Union economy

$2 billion annually [56]. In the United States, > 2 million people suffer from the antibiotic resistant bacterial infections, which causes 23,000 deaths as a direct consequent. Bacteria can be intrinsically resistant to certain antibiotics but can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer. In this chapter, each type of mechanism of bacterial resistance to antibiotics will be introduced.

1.2.1 Prevention of access to target

Reduced permeability. Compared with Gram-positive bacteria, Gram-negative bacteria are intrinsically less permeable to many antibiotics as there is a permeability barrier on its outer membrane. In most Enterobacteriaceae, hydrophilic antibiotics are able to cross the outer membrane by diffusing through the outer membrane porin proteins, including OmpF and OmpC of E. Coli., which are considered via a non-specific pathway [58-59]. Herein, reducing the permeability of the outer membrane or hampering the entry of antibiotics into bacteria inside can be achieved by down-regulating the activity and expression of porins or by the replacement of porins with more selective channels. Recent work reported that reductions in porins expression could significantly cause resistance to antibiotics, which were usually by enzymatic degradation [60-64]. For example, it was found that bacteria would be evolved to be resistance to carbapenems, one of the most powerful antibiotics used in clinic, if mutations reduce porin production or if mutant porin alleles are present [65]. This selective pressure exerted by carbapenems to cause mutations in porin genes and its regulation on porin expression has been demonstrated by

the rapid accumulation of genes mutation in bacteria after the carbapenem exposure [66, 67].

Increased efflux. Bacterial efflux pumps actively transport many types of antibiotics out of the bacterial cell, and they are the major contributors to the intrinsic resistance of Gram-negative bacteria to many antibiotics. Once it is overexpressed, efflux pumps will confer high levels of resistance to clinically useful antibiotics. Bacterial efflux is consisted with different types of pumps; some of them have narrow substrate specificity, but many others can transport a wide range of structurally dissimilar substrates which are well known as multidrug resistance (MDR) efflux pumps. Notably, MDR genes can be mobilized onto plasmids and transferred between bacteria, even though all bacteria carry these genes encoding MDR efflux pumps on their chromosomes [68]. The high-level expression of efflux genes found in multidrug resistance bacteria often results from mutations in the regulatory network controlling efflux pumps expression. These mutations can be within a local repressor, a global transcription factor or intergenic sites that increase the expression of pump genes or their regulators. Increased expression of efflux pumps can also be seen as a consequence in response to environmental signals and in conditions where their functions are required. For example, the *acrAB* genes in *E. coli* are up-regulated by small molecules which could be encountered during the infection, such as indole and bile [69-70].

1.2.2 Changes in antibiotic targets by mutations

Most antibiotics prevent the normal activity of the target through a specific binding with high affinity. Thereby, changes to the target structure that prevent efficient antibiotic binding, but that still enable the target to carry out its normal function, can confer resistance. During the course of infection, there are large and diverse population of bacteria, and if a single point mutation in the gene encoding an antibiotic target can confer resistance to the antibiotic, strains with this mutation can then proliferate. The genes that encode the targets of some antibiotics exist in multiple copies [71-73]. Transformation is a pathway that make bacteria uptake DNA from the environment and confer antibiotic resistance by target protein modification through the gene mutations. For example, penicillin resistance in *S. pneumoniae* is conferred by penicillin-binding protein genes (pbp) encoding penicillin-insensitive enzymes. Acquisition of a gene homologous to the original target is another example that exposure of methicillin induces the formation of resistant of *S. aureus* (MRSA), in which methicillin resistance is conferred by acquisition of the staphylococcal cassette chromosome mec (SCCmec) element. It carries the *mecA* gene that encodes the β-lactam insensitive protein PBP2α, and this protein enables cell wall biosynthesis to occur despite the native PBP being inhibited in the presence of antibiotics [74].

1.2.3 Modification of targets

Modification of targets in bacterial cells can also confer an effective means of resistance to antibiotics which does not have a requirement for the genes mutations encoding the target molecules. Most recently, it has been found that protection of targets is a clinically relevant mechanism of resistance for some important antibiotics. For example, specifically methylating A2503 in the 23S rRNA induced by chloramphenicol-florenicol resistance methyltransferase could confer resistance to a wide range of antibiotics that have targets near this site, including phenicols, pleuromutilins, streptogramins, linosamides and oxazolidonoes, *et al.* [75].

1.2.4 Direct modification of antibiotics

In addition to preventing antibiotics from entering bacteria cells or altering antibiotics targets, bacteria can destroy or modify antibiotics, thereby resisting their action.

Inactivation of antibiotics by hydrolysis. One of major mechanism of antibiotic resistance is the enzyme-catalyzed modification of antibiotics [76]. Since the first use of antibiotic penicillinase (a β-lactamase) in 1940, a large number of enzymes have been identified which can modify or degrade different classes of antibiotics, including β-lactams,

aminoglycosides, phenicols and macrolides, are hydrolysed by a diverse range of β-lactamases [77-80].

Inactivation of antibiotic by transfer of a chemical group. Bacterial enzymes can add extra chemical groups to antibiotic molecules on vulnerable sites causing antibiotic resistance through the prevention of binding of antibiotics to its target protein as a consequence of steric hindrance. Various chemical groups can be transferred, including ribitoyl groups, phosphate, acyl, and nucleotidyl groups, and these enzymes form a large and diverse family of antibiotic resistance enzymes [135]. For example, aminoglycoside antibiotics are particularly susceptible to being modified because they are large molecules with many exposed hydroxyl and amide groups. Once these groups are modified, bacteria will be evolved to high level of resistance. Currently, there are three main classes of aminoglycoside modifying enzymes have been found, including acetyltransferase, phosphotransferases and nucleotidyltransferases, which can be evolutionarily diverse and vary in in the aminoglycosides [86-88].

1.3 Bacterial tolerance to antibiotics

1.3.1 Antibiotic tolerance

Besides persistence and resistance to antibiotics, it has long been realized that other mechanisms can help bacteria survive antibiotic exposure. For example, bacteria can survive bactericidal antibiotics via a “transient dormancy” which requires active growth for killing. This property is known as “tolerance” now. In addition, many bacterial infections are able to exist in the host for long periods of time, even during the antibiotic treatment. Several factors are considered to the establishment and maintenance of persistent infections, in which another phenomenon gaining recognition for its role is the presence of tolerant cells that is distinguishable from resistant cells (Table 1.1). Tolerance cause antibiotic treatment failure, are selected for following repeated doses of antibiotics [89, 90] and have been shown to lead to the emergence of antibiotic resistance [91, 92].

Unlike resistance, tolerance is an extension of the period of time that bacteria can survive in lethal concentrations of an antibiotic before succumbing to its effects [94]. Although tolerance seems less advantages than resistance, this is only true when long-term exposure to a drug is considered. Under changing conditions, however, tolerance may have a strong advantage, which could have a profound impact on the evolutionary outcome [95, 96]. For example, it has been shown that the extension of lag-time evolves fast under

intermittent antibiotic treatments and confers high tolerance [97]. Such tolerance facilitated the evolution of resistance, because tolerance mutations that conferred partial resistance could lead to full resistance by additional mutations [98].

Table 1.1 Definition of field-specific terms [93]

Field-specific terms	*Phenotypic Definition*
Persistence	The ability of bacteria to remain viable in the host for a prolonged period of time. Not to be confused with bacterial persisters.
Bacterial persisters	Cells that represent a subset of antibiotic tolerance; persisters are a subpopulation of slow-growing or growth-arrested bacterial cells that have a decreased susceptibility to killing by bactericidal antibiotics within an otherwise susceptible clonal population, owing to a low target activity or low antibiotic uptake that is induced by stress.
Antibiotic resistance	The ability of a population of bacteria to grow in the presence of antibiotics, owing to an acquired genetic modification that enables the degradation or export of the antibiotic, or modification of the antibiotic target.
Phenotypic resistance	form of antibiotic resistance; the ability of a subpopulation of bacterial cells to grow in the presence of antibiotics within a clonal and otherwise susceptible population, owing to phenotypic variation that enables the degradation or export of the antibiotic, or modification of the antibiotic target.
Antibiotic tolerance	The reduced susceptibility of a population of bacteria to killing by bactericidal antibiotics, owing to low target activity or low drug uptake, often associated with slow growth or reduced metabolism induced by stress.

1.3.2 Quantification of antibiotic tolerance and persistence

Tolerance is actually a poorly characterized phenomenon and is seldom taken into account explicitly in healthcare. The MDK_{99} - the minimum duration for killing 99% of the bacteria - can be defined when the killing rate reaches saturation at high concentration (Fig. 1.3). For characterizing the persistence of a bacterial population, a similar indicator such as MDK99.99, can be used (Fig. 1.3). However, if the persistence level itself is needed, the fraction of the tolerant subpopulation should be measured by extrapolating to slower killing curve to the initial measurement. More details for tolerance and persistence measurement will be introduced in following chapters 3, 4 and 5.

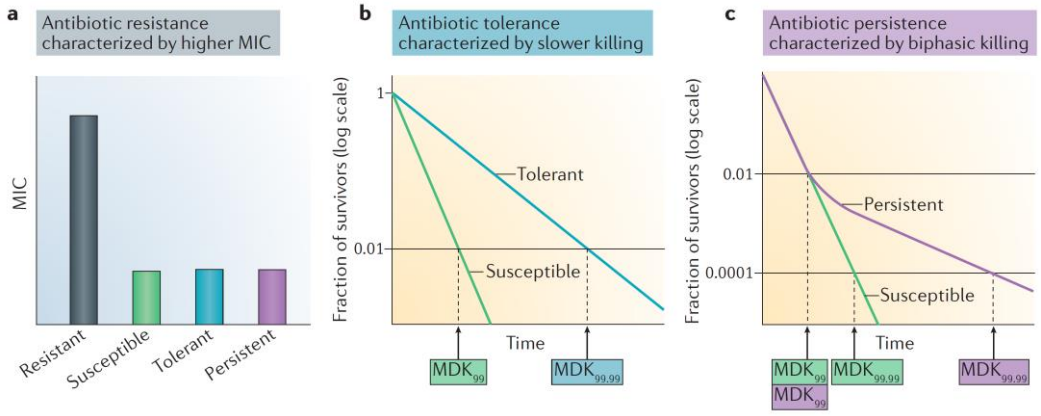

Fig. 1.3 Antibiotic resistance, tolerance and persistence [99]. *Resistance, tolerance and persistence are distinct responses to antibiotic treatment that lead to increased survival compared with susceptible cells [99]. (A) To inhibit the growth of resistant bacteria, a substantially higher minimum inhibitory concentration (MIC) of the antibiotic is needed than for susceptible bacteria. Notably, persistence and tolerance do not lead to an increase in the MIC compared with susceptible bacteria. (B) By contrast, tolerance increases the minimum duration for killing (MDK; for example, for 99% of bacterial cells in the population (*MDK_{99}*)) compared with susceptible bacteria. (C) Persistence leads to a similar MIC and a similar initial killing of the bacterial population compared with susceptible bacteria; however, the MDK for 99.99% of bacterial cells in the population (*$MDK_{99.99}$*) can be substantially higher owing to the survival of the persister cells [99].*

1.4 Evolution under antibiotics treatment: Interplay between antibiotic persistence, tolerance, and resistance

Most of the antibiotic induced resistant evolutionary experiments utilize a constant exposure of drug starting with an initial lower dose (below MIC) and a gradual daily increased doses to bacterial populations until it reaches to a higher MIC confirming the formation of resistance. However, persistent or tolerant cells can mainly be selected by the transit exposure of antibiotics since they usually stop growing under antibiotics treatments above the MIC and have an advantage only under transient treatments. It is notably that many drugs show intermittent pharmacokinetics in patients rather than constant concentrations compared to *in vitro* experiments. The achievement of antibiotics treatment tends to reach a practical concentration above the MIC for at least 50%, whereas this cannot be always achieved in patients. Thus, it is interested to investigate the evolution under intermittent antibiotic exposures and to understand the mechanisms driving the evolution *in vivo* and *in vitro*.

1.4.1 Evolution of triggered persistence

Since the days of Jacques Monod (1949), who defined the exponential phase of bacterial growth and the way to measure it precisely, microbiological studies have been primarily focusing on bacteria during this period. However, bacteria in natural environments are

rarely found growing in the exponential growth phase [100]. Pathogenic bacteria are usually considered harmful when they multiply rapidly. Not surprisingly, most current detection techniques for pathogens in the clinical environment target the fastest growing bacteria, and antibiotics are selected for their ability to kill rapidly growing strains. Understanding the way bacteria evolve under drugs administered during their stationary phase is an important and overlooked question. In the experiments of van den Bergh et al., bacteria were exposed to antibiotic only after having reached stationary phase. After treating the stationary culture with antibiotic for several hours, the antibiotic was washed away and the culture regrown to stationary phase again [101]. This work showed that mutations that elevate the level of tolerance and persistence rapidly occur also in this protocol. A similar exposure protocol led to a mutation resulting in high tolerance to daptomycin in *Staphylococcus aureus* [102]. Interestingly, the mutations identified in the experiments of van den Bergh *et al*. had no overlap with those previously identified in the evolution experiments [103]. Whether this lack of overlap is due to the difference in the type of antibiotic classes used or to the different type of tolerance remains to be determined.

1.4.2 Evolution of drug induced tolerance

A stationary phase trigger is required for the tolerant or persistent phenotype. However, certain antibiotics have been shown to be the trigger for tolerance [104-106]. In cyclic

exposure of *Streptococcus gordonii* to penicillin, Entenza *et al.* isolated high-tolerant mutants [107]. Analysis of the way these mutants survive the antibiotics revealed that instead of causing lysis, as in the wild-type strain, the drug was inducing only growth arrest, which made the bacteria highly tolerant to penicillin. These lysis-defective mutants behaved under the bactericidal drug as if it were bacteriostatic. Similar induction of tolerance or persistence by the antibiotic was observed for fluoroquinolone in *E. coli* [108].

During the 1970s, tolerant pathogenic bacteria were reported in clinical studies [109, 110], especially in deep-rooted and chronic infections. These findings led to a burst of tolerance research during the next decade that identified tolerance in over 20 species, suggesting it to be a common feature in clinical bacterial pathogens [111, 112]. However, an important question that has been difficult to answer for a long time is whether the tolerant phenotype has clinical implications or not? In 2014, a clinical work analyzed the cure rates of treatments with bacteriostatic and bactericidal drugs, and reported no statistically meaningful difference [113]. However, these data did not focus on the infections where bactericidal effect was shown to be crucial, namely infections in immune-compromised hosts, or in niches where the immune system was ineffective [113]. It is likely that in many infections, tolerance does not play a major role. With whole genome sequencing techniques, the evolution of pathogenic bacteria in the patients could be tracked. Through a series of pioneering research, evolution of tolerance was identified in patients with

chronic infection [114], compromised immune systems [115], and biofilm-associated diseases [116]. These findings suggest that tolerance could evolve in the clinic, especially when the infection is difficult to eradicate. Thus, monitoring the evolution of tolerance might be important for these patients, and update the treatment accordingly with anti-tolerance antibiotics.

1.4.3 Antibiotic tolerance and persistence promote the evolution of resistance

Most of the resistance evolution experiments were performed under gradually growing concentrations, where resistance mutations could gradually accumulate and reach a high resistance [117, 118]. To determine whether resistance establishes slower or faster on the background of tolerant strains, it is subjected to the intermittent antibiotic treatment in parallel *E. coli* strains and their evolved respective tolerant mutant strains [119, 120]. MICs of each population were measure at each cycle. It is found that the subsequent resistance evolution could be promoted by the tolerance in bacterial populations under cyclic antibiotic treatments. At concentrations above the MPC, the evolution of tolerance can lead to the fixation of partial resistance mutations that significantly elevate the probability of full resistance. Thus, tolerance mutations pave the way for the rapid subsequent evolution of resistance.

1.5 Thesis aims and outlines

Knowing when and how antibiotics trigger the evolution of persistence, tolerance, and resistance is crucial for drug prescriptions and development. In this thesis, the author will systematically introduce the research results on how to induce a superfast evolution of resistance to β-lactams antibiotics, and what the role of various genes are participating in this process.

In particular, chapter three aimed to understand the role of *recA* in the evolution of bacterial resistance to β-lactams antibiotics. *recA* is well-known to be involved in the induction of SOS response, which is required for the evolution of resistance to fluoroquinolones antibiotics, but its action is datable now.

Chapter four is aim at further understanding the role of SOS response in the evolution of bacterial resistance. In this chapter, super resolution microscopy will be applied to directly observe the induction and activation of SOS response during the treatment of antibiotics.

Chapter five aimed to investigate the mechanism for the evolution of resistance induced by single exposure of β-lactams antibiotics. Whole genome sequencing will be explored for all *E. coli* strains. It is of significance that an uncharacterized gene *pinR* is first to be

reported in this chapter which plays a decisive role in the evolution of resistance to β-lactams antibiotics.

Finally, chapter six aimed to understand a transient resistance induced by exposure of β-lactams antibiotics and ribosome inhibitors.

By identifying and analyzing the role of *recA* and *pinR*, this work first aimed to provide a new model for the superfast evolution of resistance to β-lactams antibiotics, and second, it aimed to demonstrate the potential drug target for future drug development studies.

1.6 Reference

1. World Health Organization (WHO). “Antimicrobial resistance: Global report on surveillance 2014 (WHO, 2014).
2. M. Fauvart, V. N. De Groote, J. Michiels. Role of persister cells in chronic infections: Clinical relevance and perspectives on anti-persister therapies. J. Med. Microbiol. 60, 699–709 (2011).
3. N. Q. Balaban, K. Gerdes, K. Lewis, J. D. McKinney. A problem of persistence: Still more questions than answers? Nat. Rev. Microbiol. 11, 587–591 (2013).
4. B. R. Levin, D. E. Rozen. Non-inherited antibiotic resistance. Nat. Rev. Microbiol. 4, 556–562 (2006).
5. K. Lewis. Persister cells. Annu. Rev. Microbiol. 64, 357–372 (2010).
6. N. Q. Balaban, J. Merrin, R. Chait, L. Kowalik, S. Leibler. Bacterial persistence as a phenotypic switch. Science 305, 1622–1625 (2004).
7. T. Bjarnsholt. The role of bacterial biofilms in chronic infections. APMIS Suppl. 121, 1–51 (2013).
8. D. Lebeaux, J. M. Ghigo, C. Beloin. Biofilm-related infections: Bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol. Mol. Biol. Rev. 78, 510–543 (2014).
9. M. G. Blango, M. A. Mulvey. Persistence of uropathogenic Escherichia coli in the face of multiple antibiotics. Antimicrob. Agents Chemother. 54, 1855–1863 (2010).
10. K. N. Adams et al. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell 145, 39–53 (2011).
11. B. Claudi et al., Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. Cell 158, 722–733 (2014).
12. S. Helaine et al., Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science 343, 204–208 (2014).

13. G. Manina, N. Dhar, J. D. McKinney, Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell Host Microbe 17, 32–46 (2015).
14. P. Kaiser et al., Cecum lymph node dendritic cells harbor slow-growing bacteria phenotypically tolerant to antibiotic treatment. PLOS Biol. 12, e1001793 (2014).
15. M. A. Schumacher et al., HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature 524, 59–64(2015).
16. J. E. Michiels, B. Van den Bergh, N. Verstraeten, M. Fauvart, J. Michiels, In vitro emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. Antimicrob. Agents Chemother. 60, 4630–4637 (2016).
17. H. S. Moyed, K. P. Bertrand, hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J. Bacteriol. 155, 768–775 (1983).
18. H. L. Torrey, I. Keren, L. E. Via, J. S. Lee, K. Lewis, High persister mutants in Mycobacterium tuberculosis. PLOS ONE 11, e0155127 (2016).
19. B. Van den Bergh et al., Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat. Microbiol. 1, 16020 (2016).
20. K. Stepanyan et al., Fitness trade-offs explain low levels of persister cells in the opportunistic pathogen Pseudomonas aeruginosa. Mol. Ecol. 24, 1572–1583 (2015).
21. A. Slattery, A. H. Victorsen, A. Brown, K. Hillman, G. J. Phillips, Isolation of highly persistent mutants of Salmonella enterica serovar Typhimurium reveals a new toxin-antitoxin module. J. Bacteriol. 195, 647–657 (2013). pmid: 23204462
22. L. R. Mulcahy, J. L. Burns, S. Lory, K. Lewis, Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. J. Bacteriol. 192, 6191–6199 (2010).
23. L. W. Goneau et al., Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. Antimicrob. Agents Chemother. 58, 2089–2097 (2014).

24. A. A. Al Mamun et al., Identity and function of a large gene network underlying mutagenic repair of DNA breaks. Science 338, 1344–1348 (2012).
25. N. R. Cohen, M. A. Lobritz, J. J. Collins, Microbial persistence and the road to drug resistance. Cell Host Microbe 13, 632–642 (2013).
26. M. A. Kohanski, D. J. Dwyer, J. J. Collins, How antibiotics kill bacteria: From targets to networks. Nat. Rev. Microbiol. 8, 423–435 (2010).
27. J. Bigger, Treatment of staphylococcal infections with penicillin by intermittent sterilisation. Lancet 244, 497–500 (1944).
28. J. T. Lennon, S. E. Jones, Microbial seed banks: The ecological and evolutionary implications of dormancy. Nat. Rev. Microbiol. 9, 119–130 (2011).
29. O. Gefen, N. Q. Balaban, The importance of being persistent: Heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol. Rev. 33, 704–717 (2009).
30. D. K. Fung, E. W. Chan, M. L. Chin, R. C. Chan, Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob. Agents Chemother. 54, 1082–1093 (2010).
31. S. M. Amato, M. P. Brynildsen, Persister heterogeneity arising from a single metabolic stress. Curr. Biol. 25, 2090–2098 (2015). pmid: 26255847
32. E. Maisonneuve, L. J. Shakespeare, M. G. Jørgensen, K. Gerdes, Bacterial persistence by RNA endonucleases. Proc. Natl. Acad. Sci. U.S.A. 108, 13206–13211 (2011).
33. N. Verstraeten et al., Obg and membrane depolarization are part of a microbial bet-hedging strategy that leads to antibiotic tolerance. Mol. Cell 59, 9–21 (2015).
34. T. Dörr, M. Vulić, K. Lewis, Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLOS Biol. 8, e1000317 (2010).
35. Y. Kim, T. K. Wood, Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in Escherichia coli. Biochem. Biophys. Res. Commun. 391, 209–213 (2010).

36. O. Fridman, A. Goldberg, I. Ronin, N. Shoresh, N. Q. Balaban, Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513, 418–421 (2014).
37. B. Stewart, D. E. Rozen, Genetic variation for antibiotic persistence in Escherichia coli. Evolution 66, 933–939 (2012).
38. N. Hofsteenge, E. van Nimwegen, O. K. Silander, Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of E. coli. BMC Microbiol. 13, 25 (2013).
39. T. Vogwill, A. C. Comfort, V. Furió, R. C. MacLean, Persistence and resistance as complementary bacterial adaptations to antibiotics. J. Evol. Biol. 29, 1223–1233 (2016).
40. M. Arnoldini, R. Mostowy, S. Bonhoeffer, M. Ackermann, Evolution of stress response in the face of unreliable environmental signals. PLOS Comput. Biol. 8, e1002627 (2012).
41. E. Maisonneuve, M. Castro-Camargo, K. Gerdes, (p)ppGpp controls bacterial persistence by stochastic induction of toxin-antitoxin activity. Cell 154, 1140–1150 (2013).
42. D. Shah et al., Persisters: A distinct physiological state of E. coli. BMC Microbiol. 6, 53 (2006).
43. O. Kotte, B. Volkmer, J. L. Radzikowski, M. Heinemann, Phenotypic bistability in Escherichia coli's central carbon metabolism. Mol. Syst. Biol. 10, 736 (2014).
44. Y. Wu, M. Vulić, I. Keren, K. Lewis, Role of oxidative stress in persister tolerance. Antimicrob. Agents Chemother. 56, 4922–4926 (2012).
45. S. H. Hong, X. Wang, H. F. O'Connor, M. J. Benedik, T. K. Wood, Bacterial persistence increases as environmental fitness decreases. Microb. Biotechnol. 5, 509–522 (2012).

47. V. Hauryliuk, G. C. Atkinson, K. S. Murakami, T. Tenson, K. Gerdes, Recent functional insights into the role of (p)ppGpp in bacterial physiology. Nat. Rev. Microbiol. 13, 298–309 (2015).
48. U. Mechold, K. Potrykus, H. Murphy, K. S. Murakami, M. Cashel, Differential regulation by ppGpp versus pppGpp in Escherichia coli. Nucleic Acids Res. 41, 6175–6189 (2013).
49. U. Kanjee, K. Ogata, W. A. Houry, Direct binding targets of the stringent response alarmone (p)ppGpp. Mol. Microbiol. 85, 1029–1043 (2012).
50. D. Nguyen et al., Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334, 982–986 (2011).
51. D. Viducic et al., Functional analysis of spoT, relA and dksA genes on quinolone tolerance in Pseudomonas aeruginosa under nongrowing condition. Microbiol. Immunol. 50, 349–357 (2006).
52. A. L. Spoering, K. Lewis, Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J. Bacteriol. 183, 6746–6751 (2001).
53. P. S. Stewart et al., Contribution of stress responses to antibiotic tolerance in Pseudomonas aeruginosa biofilms. Antimicrob. Agents Chemother. 59, 3838–3847 (2015).
54. S. P. Bernier et al., Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. PLOS Genet. 9, e1003144 (2013).
55. A. Boehm et al., Second messenger signalling governs Escherichia coli biofilm induction upon ribosomal stress. Mol. Microbiol. 72, 1500–1516 (2009).
56. Walker, D. & Fowler, T. Annual Report of the Chief Medical Officer: Volume Two, 2011: Infections and the Rise of Antimicrobial Resistance (Department of Health, 2011).
57. World Economic Forum. Global Risks 2013 - Eighth Edition, 2013-eighth-edition (2013).
58. World Economic Forum. Global Risks 2014 Report (2014).

59. Kojima, S. & Nikaido, H. Permeation rates of penicillins indicate that Escherichia coli porins function principally as nonspecific channels. Proc. Natl Acad.Sci. USA 110, E2629–E2634 (2013).

60. Vargiu, A. V. & Nikaido, H. Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. Proc. Natl Acad. Sci. USA 109, 20637–20642 (2012).

61. Tran, Q. T., Williams, S., Farid, R., Erdemli, G. & Pearlstein, R. The translocation kinetics of antibiotics through porin OmpC: insights from structure-based solvation mapping using WaterMap. Proteins 81, 291–299 (2013).

62. Tamber, S. & Hancock, R. E. On the mechanism of solute uptake in Pseudomonas. Front. Biosci. 8, s472–s483 (2003).

63. Baroud, M. et al. Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. Int. J. Antimicrob. Agents 41, 75–79 (2013).

64. Lavigne, J. P. et al. An adaptive response of Enterobacter aerogenes to imipenem: regulation of porin balance in clinical isolates. Int. J. Antimicrob. Agents 41, 130–136 (2013).

65. Poulou, A. et al. Outbreak caused by an ertapenemresistant, CTX-M-15-producing Klebsiella pneumonia sequence type 101 clone carrying an OmpK36 porin variant. J. Clin. Microbiol. 51, 3176–3182 (2013).

66. Wozniak, R. A. & Waldor, M. K. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nature Rev. Microbiol. 8, 552–563 (2010).

67. Wozniak, A. et al. Porin alterations present in noncarbapenemase- producing Enterobacteriaceae with high and intermediate levels of carbapenem resistance in Chile. J. Med. Microbiol. 61, 1270–1279 (2012).

68. Novais, Â. et al. Spread of an OmpK36-modified ST15 Klebsiella pneumoniae variant during an outbreak involving multiple carbapenem-resistant Enterobacteriaceae species and clones. Eur. J. Clin. Microbiol. Infecti. Dis. 31, 3057–3063 (2012).

69. Tangden, T., Adler, M., Cars, O., Sandegren, L. & Lowdin, E. Frequent emergence of porin-deficient subpopulations with reduced carbapenem susceptibility in ESBL-producing Escherichia coli during exposure to ertapenem in an in vitro pharmacokinetic model. J. Antimicrob. Chemother. 68, 1319–1326 (2013).

70. Dolejska, M., Villa, L., Poirel, L., Nordmann, P. &Carattoli, A. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance nodulation cell division/multidrug efflux pump. J. Antimicrob. Chemother. 68, 34–39 (2013).

71. Hirakawa, H., Inazumi, Y., Masaki, T., Hirata, T. & Yamaguchi, A. Indole induces the expression of multidrug exporter genes in Escherichia coli. Mol. Microbiol. 55, 1113–1126 (2005).

72. Nikaido, E. et al. Effects of indole on drug resistance and virulence of Salmonella enterica serovar Typhimurium revealed by genome-wide analyses. Gut Pathog. 4, 5 (2012).

73. Nikaido, E., Shirosaka, I., Yamaguchi, A. & Nishino, K. Regulation of the AcrAB multidrug efflux pump in Salmonella enterica serovar Typhimurium in response to indole and paraquat. Microbiology 157, 648–655 (2011).

74. Billal, D. S., Feng, J., Leprohon, P., Legare, D. & Ouellette, M. Whole genome analysis of linezolid resistance in Streptococcus pneumoniae reveals resistance and compensatory mutations. BMC Genomics 12, 512 (2011).

75. Gao, W. et al. Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog. 6, e1000944 (2010).

76. Leclercq, R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. 34, 482–492 (2002).

77. Unemo, M. et al. High-level cefixime- and ceftriaxoneresistant Neisseria gonorrhoeae in France: novel penA mosaic allele in a successful international clone causes treatment failure. Antimicrob. Agents Chemother. 56, 1273–1280 (2012).

78. Long, K. S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S. & Vester, B. The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. Antimicrob. Agents Chemother. 50, 2500 2505 (2006).

79. Abraham, E. P. & Chain, E. An enzyme from bacteria able to destroy penicillin. 1940. Rev. Infect. Dis. 10, 677–678 (1988).

80. Livermore, D. M. Defining an extended-spectrum betalactamase. Clin. Microbiol. Infect. 14 (Suppl. 1), 3–10 (2008).

81. Nordmann, P., Poirel, L., Walsh, T. R. & Livermore, D. M. The emerging NDM carbapenemases. Trends Microbiol. 19, 588–595 (2011).

82. Voulgari, E., Poulou, A., Koumaki, V. & Tsakris, A. Carbapenemase-producing Enterobacteriaceae: now that the storm is finally here, how will timely detection help us fight back? Future Microbiol. 8, 27–39 (2013).

83. Woodford, N., Turton, J. F. & Livermore, D. M. Multiresistant Gram-negative bacteria: the role of highrisk clones in the dissemination of antibiotic resistance. FEMS Microbiol. Rev. 35, 736–755 (2011).

84. Johnson, A. P. & Woodford, N. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. J. Med. Microbiol. 62, 499–513 (2013).

85. Queenan, A. M. & Bush, K. Carbapenemases: the versatile β-lactamases. Clin. Microbiol. Rev. 20, 440–458 (2007).

86. Queenan, A. M., Shang, W., Flamm, R. & Bush, K. Hydrolysis and inhibition profiles of β-lactamases from molecular classes A to D with doripenem, imipenem, and meropenem. Antimicrob. Agents Chemother. 54, 565–569 (2010).

87. Tzouvelekis, L. S., Markogiannakis, A., Psichogiou, M., Tassios, P. T. & Daikos, G. L. Carbapenemases in Klebsiella pneumoniae and other Enterobacteriaceae: an evolving crisis of global dimensions. Clin. Microbiol. Rev. 25, 682–707 (2012).

88. Wright, G. D. Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv. Drug Delivery Rev. 57, 1451–1470 (2005).

89. Norris, A. L. & Serpersu, E. H. Ligand promiscuity through the eyes of the aminoglycoside N3 acetyltransferase IIa. Protein Sci. 22, 916–928 (2013).

90. Romanowska, J., Reuter, N. & Trylska, J. Comparing aminoglycoside binding sites in bacterial ribosomal RNA and aminoglycoside modifying enzymes. Proteins 81, 63–80 (2013).

91. Qin, S. et al. Identification of a novel genomic island conferring resistance to multiple aminoglycoside antibiotics in Campylobacter coli. Antimicrob. Agents Chemother. 56, 5332–5339 (2012).

92. Mechler, L. et al. A novel point mutation promotes growth phase-dependent daptomycin tolerance in Staphylococcus aureus. Antimicrob. Agents Chemother. 59, 5366–5376 (2015).

93. Van den Bergh, B. et al. Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat. Microbiol. 1, 16020 (2016).

94. Cohen, N. R., Lobritz, M. A. & Collins, J. J. Microbial persistence and the road to drug resistance. Cell Host Microbe 13, 632–642 (2013).

95. Levin-Reisman, I. et al. Antibiotic tolerance facilitates the evolution of resistance. Science 355, 826–830 (2017).

96. Robert, A., Bridget, Gollan & Sophie Helaine. Persistent bacterial infections and persister cells. Nature Reviews Microbiology 15, 453-464 (2017).

97. Handwerger, S., & A. Tomasz. Antibiotic tolerance among clinical isolates of bacteria. Annu. Rev. Pharmacol. Toxicol. 25, 349-380 (1985).

98. Kussell, E., R. Kishony, & S. Leibler. Bacterial persistence: a model of survival in changing environments. Genetics. 169, 1807– 1814 (2005).

99. Bala. Bergkessel, M., Basta, D. W., & Newman, D. K. (2016). The physiology of growth arrest: Uniting molecular and environmental microbiology. Nature Reviews. Microbiology, 14, 549–562.

101. Van den Bergh, B., et al. (2016). Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nature Microbiology, 1, 16020.

102. Mechler, L., et al. (2015). A novel point mutation promotes growth phase-dependent daptomycin tolerance in Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 59, 5366–5376.

103. Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., & Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature, 513, 418–421.

104. Dörr, T., Vulić, M., & Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biology, 8, e1000317.

105. Audrain, B., et al. (2013). Induction of the Cpx envelope stress pathway contributes to Escherichia coli tolerance to antimicrobial peptides. Applied and Environmental Microbiology, 79, 7770–7779.

106. Moreillon, P., Tomasz, A., & Tomasz, A. (1988). Penicillin resistance and defective lysis in clinical isolates of pneumococci: Evidence for two kinds of antibiotic pressure operating in the clinical environment. The Journal of Infectious Diseases, 157, 1150–1157.

107. Entenza, J. M., Caldelari, I., Glauser, M. P., Francioli, P., & Moreillon, P. (1997). Importance of genotypic and phenotypic tolerance in the treatment of experimental endocarditis due to Streptococcus gordonii. The Journal of Infectious Diseases, 175, 70–76.

108. Dörr, T., Vulić, M., & Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biology, 8, e1000317.

109. Best, G. K., Best, N. H., & Koval, A. V. (1974). Evidence for participation of autolysins in bactericidal action of oxacillin on Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 6, 825–830.

110. Sabath, L. D., Laverdiere, M., Wheeler, N., Blazevic, D., & Wilkinson, B. J. (1977). A new type of penicillin resistance of Staphylococcus aureus. Lancet, 309, 443–447.

111. Handwerger, S., & Tomasz, A. (1985). Antibiotic tolerance among clinical isolates of bacteria. Annual Review of Pharmacology and Toxicology, 25, 349–380.

112. Tomasz, A. (1985). Antibiotic tolerance among clinical isolates of bacteria. Antimicrobial Agents and Chemotherapy, 7, 368–386.

113. Nemeth, J., Oesch, G., & Kuster, S. P. (2015). Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: Systematic review and meta-analysis. The Journal of Antimicrobial Chemotherapy, 70, 382–395.

114. Mulcahy, L. R., Burns, J. L., Lory, S., & Lewis, K. (2010). Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. Journal of Bacteriology, 192, 6191–6199.

115. Honsa, E. S., et al. (2017). Rela mutant Enterococcus faecium with multiantibiotic tolerance arising in an immunocompromised host. MBio, 8, 1–12.

116. Dengler Haunreiter, V., et al. (2019). In-host evolution of Staphylococcus epidermidis in a pacemaker-associated endocarditis resulting in increased antibiotic tolerance. Nature Communications, 10, 1149.

117. Toprak, E., et al. (2012). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. Nature Genetics, 44, 101–105.

118. Baym, M., et al. (2016). Spatiotemporal microbial evolution on antibiotic landscapes. Science, 353, 1147–1151.

119. Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., & Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature, 513, 418–421.

120. Levin-Reisman, I., et al. (2017). Antibiotic tolerance facilitates the evolution of resistance. Science, 355, 826–830.

Chapter 2. Methods and Materials

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2.1 Chemicals, regents, and solutions

The chemicals and reagents used throughout this work were typically analytical grade and were purchased from Sigma, Amresco, BDH Chemicals, or Bio-rad Laboratories unless otherwise is specified. Commonly used buffers and solutions are listed in Table 2.1.

Table 2.1. Solutions and their working compositions

Buffer/Solutions	*Composition*[a]
APS	10% ammonium persulfate
EDTA	18.6% EDTA, 2% NaOH, pH 8.0
PBS	137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.0
GTE	50mM glucose, 20mM Tris-Cl_2, 10mM EDTA, pH 7.5
TE	10mM Tris-HCl, 1mM EDTA, pH 7.5
TBE	89mM Tris-HCl, 89mM boric acid, 2.5 mM EDTA, pH 8.3
TBS	50 mM Tris-HCl, 150 mM NaCl
TS	200 mM Tris-HCl, 100 mM NaCl
Coomassie Blue Stain	50% Ethanol (v/v), 10% Glacial Acetic Acid ≥ 99.85% (v/v), 0.5% Coomassie R-250 (w/v)
Destaining Buffer	20% Ethanol (v/v), 10% Glacial Acetic acid ≥ 99.85% (v/v)
SDS-PAGE loading buffer (2X)	0.62M Tris-HCL pH 6.8, 10% SDS, 20% Glycerol (v/v), 0.1% Bromophenol blue
SDS-PAGE running buffer (2X)	14.42% Glycine, 3% Tris, 1% SDS

[a] *All buffer and solutions were made up in MilliQ® purified water (MQW; Millipore) and are listed at the normal working concentration (1X) or stated otherwise. All percentages are given as weight per volume (w/v) unless stated otherwise.*

2.2 *Escherichia coli* strains and growth conditions

All *E. coli* strains used in this thesis are listed in Table 2.2. *E. coli* cells were grown on LB agar (1.5% w/v) plates at 37ºC (Table 2.3), unless stated otherwise, antibiotics (Table 2.4) were supplemented, where appropriate. Broth culture were grown in 50 ml falcon tubes or 250 ml flasks, incubated with vigorous shaking (250 rpm) at 37ºC, unless otherwise stated. Growth was monitored by recording the absorbance at 600 nm (OD_{600}) using a spectrophotometer (UV-1601 UV-visible spectrophotometer; Shimadzu). *E. coli* strains were stored as stationary phase cultures suspended in 20% (v/v) glycerol at -80ºC.

Table 2.2. *E. coli* strains used in this study

Strain	***Genotype and Relevant Characteristics***	***Source***
DH5α	F-*hsdR17 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1*	Lab Stock
MG1655	Rph1 ilvG rfb-50	Lab Stock
BW25113 (wild-type)	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Lab Stock
ΔrecA	MG1655*ΔrecA*	This study
ΔpinR	MG1655*ΔpinR*	This study
ΔrecAΔpinR	MG1655*ΔrecAΔpinR*	This study
JW0941-1 (ΔsulA)	BW25113*ΔsulA*	Lab Stock
JW0941-1 (Δymfm)	BW25113*Δymfm*	Lab Stock
JW0941-1 (ΔtolC)	BW25113*ΔtolC*	Lab Stock
EAW26 (Δ_lexA)	MG1655*ΔlexA*	Gift from Dr. Andrew Robinson (UOW)
RW1570 (lexA^{ind-})	MG1655*lexA3*	Gift from Dr. Andrew Robinson (UOW)

Table 2.3. Media used for bacterial growth

Media	*Composition*
Luria-Bertani (LB) broth	10% Tryptone, 5% Yeast Extract, 10% Sodium Chloride
M9	6% Na_2HPO_4, 3% KH_2PO_4, 1% NH_4Cl, 0.5% NaCl, 0.003% $CaCl_2$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.2% Carbon Source (Glycerol)

Table 2.4. Antibiotics used for selection in *E. coli*

Antibiotics [a]	*Working Concentration*
Ampicillin	100 µg/ml
Chloramphenicol	17 µg/ml
Kanamycin	50 µg/ml
Tetracycline	10 µg/ml
Spectinomycin	10 µg/ml

[a] *Stock solutions were made by dissolving antibiotics in either MilliQ filter sterilizing (0.2 µm filter). Antibiotic solutions were stored at -20ºC (long-term) or at 4ºC (short-term).*

2.3 Plasmids used in this study

A list of plasmids used in this thesis is described in Table 2.5.

Table 2.5. Plasmids constructed and used in this study

Plasmid	***Description***	***Source***
pJM1071*-recA-*clone	Gene *recA* cloned into the MCS of pJM1071, spectinomycin resistance	Gift from Dr. Andrew Robinson (UOW)
pKD3*-pinR-*clone	Gene *pinR* cloned into the MCS of pKD3, chloramphenicol resistance	This study
pBR322*-recA-*clone	Gene *recA* cloned into the MCS of pBR322, tetracycline resistance	This study
***pKD4*-pinR-pinQ-clone**	Genes *pinR, pinQ* cloned into the MCS of pKD4, kanamycin resistance	This study
***pJB042*-ftsZ-mEos2**	Gene *ftsZ* and superfolder *mEos2* at the N-terminal site cloned into the MCS of pCA24N, chloramphenicol resistance	[Addgene]

2.4 Growth curves

2.4.1 96-well plates

Overnight cultures grown in LB with ampicillin 100 μg/mL and 0.2% glucose were diluted to OD_{600} = 0.04 in the same media and grown to mid-exponential phase. Samples were washed in fresh LB and diluted to an OD_{600} = 0.04 in LB with either 0.2% glucose or 0.2% arabinose and ampicillin 100 μg/mL. Duplicates of each culture were aliquoted at 100μL each into a 96-well plate. The plate reader was set up to take OD_{600} measurements every 15 minutes for 6 hours, incubated at 37ºC with continuous shaking at 900 rpm. Data was exported into excel and plotted onto a semi-logarithmic graph.

2.4.2 50 ml flasks

Strain was cultured overnight in 50 mL of LB, supplemented with antibiotics if necessary, at 37ºC. Overnight cultures were diluted in 50 mL of LB (antibiotics added if necessary) to an OD_{600} = 0.01. Cultures were grown over a period of 6 hours. Growth was monitored and recorded using a spectrophotometer by removing a 1 mL aliquot, every 30 minutes. Growth curves were performed in biological triplicates. Time point and OD values were exported into excel and plotted onto a semi-logarithmic graph. This method was used to measure growth rate of mutant strains (Table 2.2) constructed in this study as well as the

number of generations needed to induce expression of genes. Mutant strains did not affect growth rate as compared to wild-type. Generation (doubling) time was calculated by determining the time (minutes) it took for the OD_{600} value to double when in exponential growth phase.

2.5 Antibiotic survival assays

Overnight cultures were used to measure the survival under antibiotic treatments. Each grown from a single colony in LB medium was diluted 1:500 in fresh medium supplemented with either 100 μg/ml ampicillin. At indicated time points, aliquots of the cultures were sampled, diluted to the appropriate dilutions and plated on LB plates. After each overnight incubation, bacterial CFUs were evaluated by plating before and after antibiotic exposure. All experiments were repeated in at least two independent experiments, unless otherwise is specified.

2.6 Antibiotic susceptibility tests

2.6.1 MIC test

Overnight cultures were used to test MIC value. Each grown from a single colony in LB medium was loaded in a 96 wells plate that is filled with fresh LB medium supplemented with increasing amounts of ampicillin from 0 to 100 μg/ml and inoculated with approx. 1×10^4 bacteria/well. The plate is incubated overnight at 37°C with shaking at 900 rpm. The MIC is the highest concentration supporting visible growth. When performing this measurement on a batch culture experiments which contains a mixture of clones, the MIC was the highest measure value rather than the average value, to reflect the MIC of the most resistant clone in the culture.

2.6.2 Disk diffusion assay

Overnight cultures were plated on LB agar plates at approx. 1×10^6 CFU/ml and left to dry for 15 minutes before placing a 10 μg ampicillin disk (Bio-rad, US) in the middle of the plate. Antibiotic susceptibility was evaluated by comparing the radius zone of inhibition.

2.6.3 MDK_{99} test

Overnight cultures were used to test MDK_{99} value. Each grown from a single colony in LB medium was loaded in a 96 wells plate that is filled with fresh LB medium supplemented with increasing amounts of ampicillin in which the lowest concentration of ampicillin was 100 µg/ml and inoculated with approx. 1×10^4 bacteria/well. Inoculation times are set so that all rows end their respective treatment at the same time. Once incubation is concluded, the plate is spun down to terminate the antibiotic exposure by washing away antibiotic remains and resuspending in fresh medium. The plate is then returned for overnight incubation. Empty wells indicate killing of >99% of the population, because growth in the well would imply that at least one bacterium. An evaluation of MDK_{99} can be read directly from the plate depending on the treatment duration at which the plateau forms.

2.7 ScanLag: High-throughput quantification of colony growth and lag time

ScanLag system is an automated method that enables lag times and growth time distributions to be assessed, which is developed by Nathalie Q. Balaban and her colleagues (Fig. 2.1).

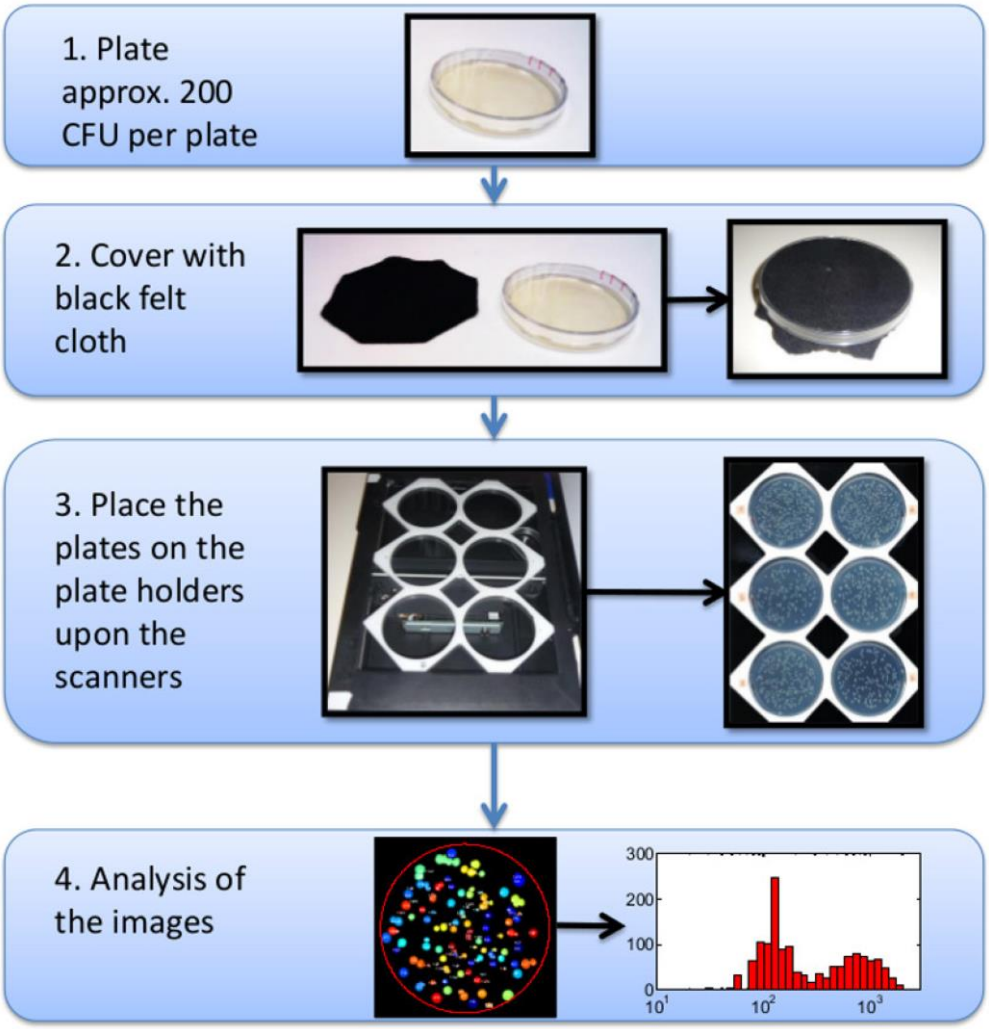


Figure 2.1 A schematic diagram of the steps needed to create a colony appearance distribution.

After each cycle, a sample of the culture was diluted serially and plated on solid LB agar medium supplemented with the appropriate selective antibiotics. The plates were placed in the ScanLag setup at 37°C. The ScanLag setup is an array of office scanners, which takes images of the plates automatically every 15 minutes, and thus monitors the

appearance of thousands of colonies. An automated image analysis application extracts the distribution of appearance time of the colonies.

2.8 Mutation frequency

Bacterial population mutation frequency was evaluated based on the approach of Delbrück-Luria Fluctuation test. 30 µl overnight cultures ($1 \times 10^4 \sim 10^6$ amounts of *E. coli* cells) were plated onto solid LB agar plates supplemented with or without 50 µg/ml ampicillin. The number of colonies appearing on the plates was used to calculate the mutation rate (Equitation 2.1).

$$R = \frac{n}{N} \times P \times 100\% \quad \textit{(Equitation 2.1)}$$

R = Mutation Frequency Rate

n = Numbers of colonies appearing on the medium containing ampicillin

N = Numbers of colonies appearing on the fresh medium

P = Population surviving rate

2.9 General DNA Methods

2.9.1 Extraction and purification of *E. coli* DNA from bacterial cultures

2.9.1.1 Chromosomal DNA

Chromosomal DNA was extracted and purified using the PureLink™ Genomic DNA mini kit (ThermoFisher Scientific) following the manufacturer's instructions. In summary, a volume of 1 mL of overnight culture of the required strain was centrifuged for 2 minutes at 10,000 g. The cell pellet was resuspended in 180 µL PureLink™ genomic digestion buffer and 20 µL of Proteinase K and incubated at 55ºC for 60 minutes until lysis was complete. A volume of 20 µL of RNase A (provided with the kit) was added to the lysate and mixed for 10 seconds on the vortex. The sample was then incubated for 2 minutes at room temperature. A volume of 200 µL of PureLink™ genomic Lysis/Binding buffer was added to the sample and mixed for 20 seconds by vortexing until a homogenous solution was obtained. 200 µL of 100% ethanol was added to the lysate and mixed well by vortexing for 5 seconds. The lysate (approximately 650 µL) was loaded onto a PureLink™ spin column in a collection tube and centrifuged at 10,000 g for 1 minute at room temperature. The flow through liquid was discarded and the column was washed twice with wash buffer 1 and wash buffer 2. The column was dried with a final spin for 3

minutes at 10,000 g. DNA was eluted using 200 µL of PureLink™ genomic elution buffer into a new 1.5 mL Eppendorf tube.

2.9.1.2 Plasmid DNA

Plasmid DNA was extracted and purified using PureLink™ Quick Plasmid Miniprep kit (ThermoFisher Scientific) following the manufacturer's instructions. In summary, 3 mL of overnight culture of *E. coli* strain harboring desired plasmid was centrifuged at 10,000 g. Cell pellet was resuspended with 250 µL of resuspension buffer. Cells were lysed by adding 250 µL of lysis buffer and mixing by gently inverting the tube. A volume of 350 µL of the precipitation buffer was added and sample mixed by inverting. The precipitate was separated from the supernatant via centrifugation at 10,000 g for 10 minutes. The supernatant was loaded onto a spin column and spun for 1 minute at 10,000 g. The column was washed using 700 µL of the wash buffer and spun at 10,000 g for 1 minute. The column was dried and excess wash buffer removed with a 2 minute spin at 10,000 g. Plasmid DNA was eluted using 50 µL of TE buffer into a new 1.5 mL Eppendorf tube.

2.9.2 Polymerase chain reaction (PCR)

PCR was used in this study for cloning genes into plasmids, confirming correct insertion of genes into plasmids and for the deletion of genes from the chromosome. Single-

stranded oligonucleotide primers for PCR were generated by IDT and supplied in a lyophilised form. Primers were dissolved in MilliQ to a final concentration of 100 μM and stored at –20°C. Primers used are listed below in relevant Tables.

2.9.2.1 PCR using Phusion® High-Fidelity DNA Polymerase

PCR with Phusion® polymerase (New England Biolabs) was used for the amplification of genes for cloning and deletions. Each reaction was set up with Phusion HF buffer (1 × v/v), dNTPs (200 μM), forward and reverse primers (0.5 μM), template DNA (100 ng), 1 unit of Phusion DNA polymerase and sterile MilliQ water to a final volume of 50 μL.

PCR reactions were subjected to the following temperature cycling using a thermocycler (Mastercycler, Eppendorf): 98ºC for 1 minute (initial denaturation of the template), followed by 30 cycles of 98ºC for 10 seconds (template denaturation), XºC for 30 seconds (primer annealing), and extension at 72ºC for Y seconds (extension), followed by a final extension step was at 72ºC for 10 minutes. XºC for each primer is listed in their respective tables, and Y was 30 seconds per kb of amplicon to be amplified.

The PCR products were analysed via agarose gel electrophoresis (Section 2.4.4) and cleaned using the PureLink™ PCR purification kit (ThermoFisher Scientific) following the manufacturer's instructions. Concentration of PCR product was determined using the

Nanodrop spectrophotometer (OD_{260} nm) (Section 2.4.3). PCR products were stored at -20ºC until further use.

2.9.2.2 PCR using Taq DNA polymerase

PCR with Taq DNA polymerase (New England Biolabs) was used for colony PCR to confirm correct insertion of gene into plasmids. A volume of 20 µL of sterile MillQ was added to each PCR tube. A colony was selected from plates and re-suspended in the water. Cells were lysed using a thermocycler, for 5 minutes at 95ºC. Samples were then placed on ice as the following reaction was set up. The following were added to each PCR tube and made to a final reaction volume of 25 µL: Thermopol buffer (1 × v/v), dNTPs (200 µM), forward and reverse primers (0.2 µM), and 1 unit of Taq DNA polymerase.

PCR reactions were subjected to the following temperature cycling using a thermocycler: 95ºC for 30 seconds (initial denaturation of the template), followed by 30 cycles of 95ºC for 15 seconds (template denaturation), XºC for 30 seconds (primer annealing), and extension at 68ºC for Y seconds (extension), followed by a final extension step was at 68ºC for 5 minutes. XºC for each primer is listed in their respective tables, and Y was 1 minute per kb to be amplified.

The PCR products were analyzed via an agarose gel electrophoresis to confirm correct insertion of DNA product into plasmid (Section 2.4.4).

2.9.3 Determining DNA concentration

Concentration of genomic and plasmid DNA was determined on the Nanodrop™ spectrophotometer. Absorbance at 260nm was blanked using either water or elution buffer. A volume of 2 μL of DNA sample was used to determine DNA concentration (ng/μL).

2.9.4 Agarose gel electrophoresis

Agarose gels were used for visualization of PCR products of the correct size and quality of either genomic or plasmid DNA. Agarose (Sigma) at 1% (w/v) was dissolved in electrophoresis buffer (TBE). Gels were cast on a horizontal slab gel apparatus (Bio-rad) with a well-forming comb in place. Gels contained 60 ng/mL of GelRed® (Biotium) to visualize the DNA under UV light. The DNA sample was prepared in 6× gel loading dye purple (New England Biolabs) and loaded into the pre-cast wells. A 1 kb DNA ladder (New England Biolabs) was used to estimate size of DNA product ranging from 500 bp to 10 kb. Agarose gels were run submerged under a volume of 1 x TBE electrophoresis buffer that lay above the surface of the gel at 90 V for approximately 1 hour. The gels

were visualized via exposure to short-wavelength UV light (254 nm) using a transilluminator (Ingenius3; Syngene) and recorded as a digital image using a charge-coupled device (CCD) camera (Synoptics CAM-FLXCM, Syngene) linked to GeneSys molecular imaging software, version 1.5.0.0 (Syngene).

2.10 Transformation of plasmid DNA into *E. coli* strains

2.10.1 Preparation of $CaCl_2$ competent *E. coli* cells

The *E. coli* strain that was to be made competent was grown overnight in LB at 37ºC with shaking at 250 rpm. The following day, cultures were diluted to Optical Density (OD_{600}) of 0.05 in 500 mL of LB. Cells were grown to an OD_{600} ranging from 0.6 to 0.8, after which they were chilled on ice for 10 minutes. Cells were centrifuged at 3,000 g at 4ºC for 10 minutes and the supernatant discarded. The pellet was resuspended in 10 mL ice cold 0.05 M $CaCl_2$ and incubated on ice for 30 min. The solution was centrifuged again at 3,000 g for 10 minutes at 4ºC. The pellet was re-suspended in 4 mL of ice cold sterile 15% glycerol (v/v) containing 0.05 M $CaCl_2$, and incubated on ice for 10 min. Cells were either used immediately or were aliquoted in 200 µL volumes into Eppendorf tubes, snap frozen using liquid nitrogen and stored at -80ºC for later use.

2.10.2 Heat-shock transformation of competent *E. coli* cells

A concentration of 50 ng (unless otherwise stated) of desired plasmid was added to 50 µL of competent cells and incubated on ice for 30 min. This was then heated shock by placing the tube into a 42ºC water bath for 90 seconds, and following to put the tubes back on ice for 5 min. After the transformation, the cells were transferred into 1 ml LB medium to recover for 1 hour at 37ºC with shaking at 250 rpm. 100µL undiluted cells were plated out onto antibiotic selective LB agar plates. The remainder of the sample was concentrated by 10 times and plated onto another plate. Plates were incubated overnight at 37ºC (unless otherwise stated).

2.10.3 Preparation of electro competent *E. coli* cells

The *E. coli* strain that was to be made electro-competent was grown overnight in LB at 37ºC with shaking at 250 rpm. The following day, cultures were diluted to Optical Density (OD_{600}) of 0.05 in 500 mL of LB. Cells were grown to an OD_{600} ranging from 0.6 to 0.8, after which they were chilled on ice for 30 minutes. Cells were centrifuged at 2,000 g at 4ºC for 10 minutes and the supernatant discarded. The pellet was resuspended in 500 mL ice cold sterile MilliQ water and centrifuged again at 2,000 g for 10 minutes at 4ºC. The pellet was re-suspended in 250 mL of ice cold sterile 10% glycerol (v/v), and centrifuged at 2,000 g for 15 minutes at 4ºC. The cells were re-suspended again in 20 mL

of 10% glycerol and centrifuged at 2,000 g for 20 minutes. Finally, the cells were resuspended in 5 mL of 10% glycerol to achieve a 100 × concentration. Cells were either used immediately for electroporation or were aliquoted in 100 µL volumes into Eppendorf tubes, snap frozen using liquid nitrogen and stored at -80ºC for later use.

2.10.4 Electroporation of competent *E. coli* cells

A concentration of 50 ng (unless otherwise stated) of desired plasmid was added to 50 µL of competent cells on ice. This was then transferred into a prechilled 0.2cm electroporation cuvette (BioRad, USA), ensuring no bubbles were produced during the process. The cells were electroporated using PowerPac Basic (BioRad, USA) electroporation machine at 25 µF (capacitance), 2.5 kV (voltage) and 200 ohm (resistance). For a successful transformation, the time constant for the pulse was between 4 to 5 milliseconds. Once electroporated, the cells were immediately transferred into 1mL of LB broth to recover for 1 hour at 37ºC. 100µL undiluted cells were plated out onto antibiotic selective LB agar plates. The remainder of the sample was concentrated by 10 times and plated onto another plate. Plates were incubated overnight at 37ºC (unless otherwise stated).

2.11 Construction of deletion mutants using lambda recombination

Lambda Red recombination (shrin 164, 165) was used to generate various gene deletion in *E. coli* strains (Table 2.2).

2.11.1 Amplification of tetracycline or chloramphenicol cassette for the deletion of *recA* or *pinR*

Genomic DNA of *E. coli* MG1655, containing insertions of a tetracycline or chloramphenicol resistance cassette to replace the open reading frame of *recA* or *pinR*, was used to make subsequent gene deletions. Primers (Table 2.6) were designed approximately 50 bp upstream and downstream to genes of interest on the chromosome, in order to amplify the tetracycline or chloramphenicol cassette as well as the flanking DNA sequence needed for homologous recombination. Phusion polymerase (NEB) was used to amplify DNA sequence (Section 2.4.2) and the reaction was cleaned up using a PureLink™ PCR purification kit (ThermoFisher Scientific) as per the manufacturer's instructions.

Table 2.6. List of primers used to delete gene *recA* or *pinR*

Gene	*Forward or Reverse*	*Sequence (5`-3`)*	*Annealing Temp (ºC)*
recA	F	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAACAAGACG AGAAACGAGAGAGGATGCTCAC	55
	R	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAA GACGTCTAAGAAACCATTATTATCATGAC	58
pinR	F	CAAGGAAGGGGGCTTGGAAGACGTAAAGCATCTCACACCGAGATTATTTT CATATGAATATCCTCCTTAGTTCCTATTC	57
	R	TTTCTGAGATGCATTATGATATGAACACCAATTTCGTATAGAGTCTCACTG AGCTGCTTCGAAGTTCCTA	55

2.11.2 Transformation of tetracycline or chloramphenicol resistance cassette and selection of successful insertion into *recA* or *pinR*

Background strain (MG1655) was made electro-competent and transformed with recombinase plasmid pKD46 and selected on LB agar plates containing 100 µg/ml ampicillin at 30ºC. The strain now containing the plasmid was made electro-competent again using LB media containing 100 µg/ml ampicillin and 0.2% arabinose at 30ºC. Amplified DNA was transformed into recipient strain by using 50 ng of DNA and 50µL of competent cells. Cells were allowed to recover in LB media for 1 hour at 30ºC. Transformation was plated onto LB agar plates containing 10 µg/mL tetracycline or 17 µg/ml chloramphenicol and incubated overnight at 37ºC.

PCR was used to confirm insertion of the tetracycline or chloramphenicol resistance cassette at the correct site on the chromosome using primers upstream and downstream to the gene of interest. PCR products were visualized on a 1% agarose gel to confirm products of the expected size (Fig 2.2 A and B).

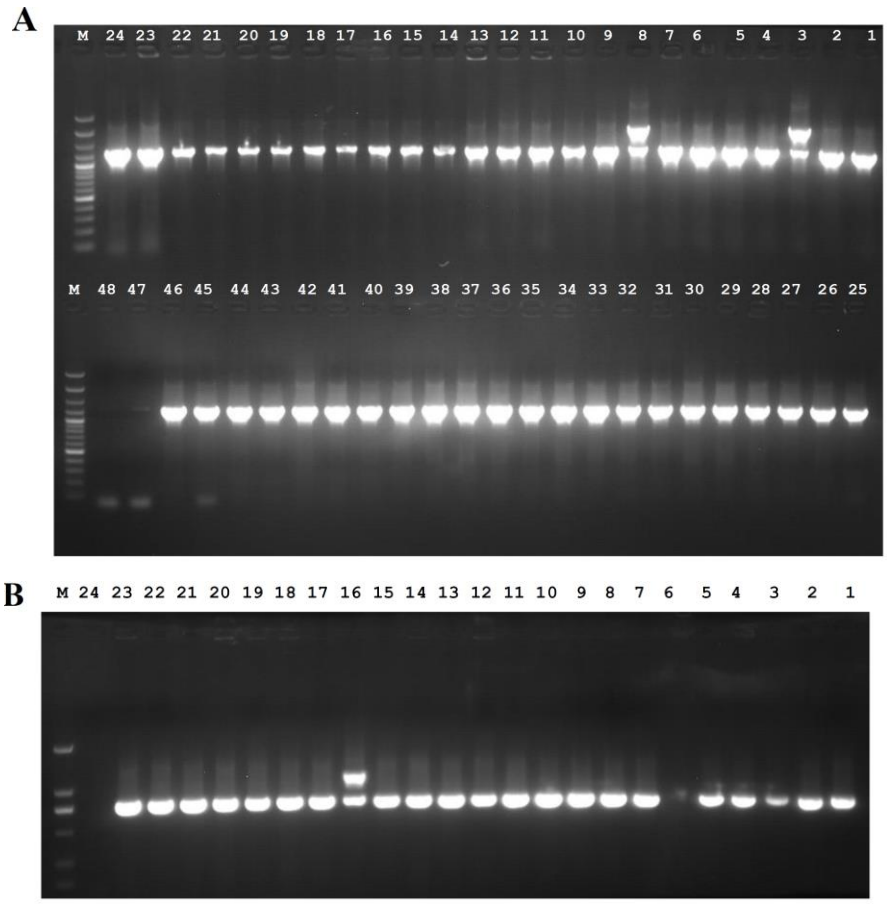


Figure 2.2 PCR to determine resistance cassette at the correct site on the chromosome. *(A) Tetracycline resistance cassette was successfully inserted into strains #3 and #8. (B) Chloramphenicol resistance cassette was successfully inserted into strain #16. DNA molecular are 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 100, 100, respectively, from top to bottom.*

The newly constructed mutant strains were cured of plasmid pKD46 through incubation of LB streak plates at 42ºC overnight. Loss of the plasmid was confirmed by lack of ampicillin sensitivity on LB agar plates.

2.11.3 Removal of resistance cassette

Mutant strains were made electro-competent and 50 μL of cells were transformed with plasmid pCP20 and incubated on 100 μg/ml ampicillin plates at 30ºC overnight. A few colonies were then re-streaked onto LB plates and incubated overnight at 42ºC. Loss of cassette and plasmid was confirmed by PCR products.

2.12 Droplet digital PCR (ddPCR) to determine gene expression levels

2.12.1 Primer design and validation

Primers (Table 2.7) were designed using OLIGO Primer Analysis Software (US). Genes of interested included, *ampC, ampD, ampE, ampG, pinR, bolA,* and *nac*. Housekeeping gene included *cysG.* Primers were validated for specificity by PCR amplification of target using MG1655 genomic DNA and confirming the presence of only one product of the correct size by 2% agarose gel electrophoresis.

Table 2.7. Primers used for ddPCR

Gene	*Forward or Reverse*	*Sequence (5`-3`)*	*Annealing Temp (°C)*
ampC	F	TTA CTT TAC CTG GGG CTA TG	59.9
	R	GTG GGA TCG CTT AAC TTG A	60.3
ampD	F	GAT CGA CGC ATT ATT CAC TG	60.9
	R	CGA AAG GAA CAT ACT GGA CTA	59.6
ampE	F	GGC GTG ACT TTT TTA CTG TT	59.6
	R	ACT TTA CCT GCG CCA ATA C	60.2
ampG	F	CAT TGG TTT CTT CTC TCT GGT	60.7
	R	AAT GGC GAC TAA TAA CAG GA	59.9
pinR	F	CTT GCT CGC CTG AAA TGT	61
	R	TTT CGG TCA GTT GTT CCA C	60.5
bolA	F	TTT TAA AGT TGT GCT GGT CAG	60.7
	R	TAG TGT AAG TAT GCA GAG CCA G	60.2
nac	F	GAA AAT AGT GGT GCA GTG CT	59.9
	R	CAA AGC CTG ACT GGA TAC AC	59.8
cysG	F	CGA AAA ACT TGA ATC ACT GC	60.6
	R	AAT GGC TTT CTG ATC GTT G	60.3

2.12.2 DNA extraction

DNA extraction is described in Chapter 2.9.1,

2.12.3 RNA extraction

A volume of 1 ml culture medium was taken from MG1655. Cells were pelleted at maximum speed in a microfuge for 2 minutes. The supernatant was discarded and the pellet was snap frozen in liquid nitrogen. Pellets were kept in the -80ºC freezer until further use.

RNA was extracted from the cell pellets using a PureLink RNA minikit (Invitrogen) as per the manufacturer's instructions. 1×10^9 log-phase bacterial cells was harvested and transferred to a microcentrifuge tube to centrifuge at 500 g at 4ºC for 5 minutes to pellet cells. 100 µl of prepared lysozyme solution (Table 2.8) was added to the cell pellet to resuspend the cells by vortexing. 0.5 µl 10% SDS was then followed to be added and the cells were incubated at room temperature for 5 minutes. After the incubation, 350 µl lysis buffer prepared with 2-m-mercaptoethanol was added and cells were vortexed to mix well. Lysate was transferred to a 1.5 ml RNase-free tube and passed 5 times through an 18-21-gauage needle attached to an RNase-free syringe. The supernatant was collected through the centrifuge at 12,000 g for 2 minutes at room temperature. 250 µl 100% ethanol was

added to each volume of bacterial cell homogenate and mixed thoroughly by vortexing to disperse any visible precipitate. Sample was then transferred to a Spin Cartridge, and centrifuged at 12,000 g for 15 seconds at room temperature. Flow-through was discarded. 700 µl wash buffer I was added to the Spin Cartridge and centrifuged at 12,000 g for 15 seconds at room temperature. Flow-through was discarded. Wash buffer II was then added with ethanol to the Spin Cartridge, and centrifuged at 12,000 g for 15 seconds at room temperature. Flow-through was discarded. After the washing, the Spin Cartridge was centrifuged at 12,000 g for 1 minute to dry the RNA attached onto the membrane to a Collection tube. 50 µl RNase-free water was followed to be added to the collection tube and all tubes were incubated at room temperature for 1 minute. All samples were centrifuged at 12,000 g for 2 minutes to collect the RNA, which was stored at -80ºC for further use.

Table 2.8. Lysozyme solution

Lysozyme	*Chemicals*
100 µl for 1×10^9 cells	10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mg mg lysozyme

2.12.4 Synthesis of complementary DNA (cDNA)

Reverse transcription (RT) of RNA to cDNA was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per the manufacturer's instructions:

Starting material: 1 ng -5 ng total RNA

Control reactions: Use 1 µl of Control RNA (50 ng/µl)

1. Mix and briefly centrifuge each component before use.
2. Prepare the RNA/primer mixture in a 0.5 ml tube as follows (Table 2.9).
3. Incubate the RNA/primer mixture at 65ºC for 5 minutes, then place on ice for at least 1 minute.
4. In a separate tube, prepare the 2X reaction mix, adding each component in the indicated order (Table 2.10).
5. Add 9 µl of the 2X reaction mix to each RNA/primer mixture from step 3, mix gently, and collect by brief centrifugation.
6. Incubate at room temperature for 2 minutes.
7. Add 1 µl of SuperScript II RT to each tube.
8. Incubate at room temperature for 10 minutes.
9. Incubate at 42 ºC for 50 minutes
10. Terminate the reaction at 70 ºC for 15 minutes. Chill on ice.
11. Collect the reaction by brief centrifugation. Add 1 µl of RNase H to each tube and

incubate for 20 minutes at 37 ºC.

Table 2.9. RT-PCR reaction mix I

Component	*Amount*
RNA	n µl
10 mM dNTP mix	1 µl
Random hexamers (50 ng/µl)	1-5 µl
DEPC-treated water	to 10 µl

Table 2.10. RT-PCR reaction mix II

Component	*1 Rxn*	*10 Rxn*
10 × RT buffer	2 µl	20 µl
25 mM $MgCl_2$	4 µl	40 µl
0.1 M DTT	2 µl	20 µl
RNaseOUT (40 U/µl)	1 µl	10 µl

2.12.5 Copy number variation analysis used by ddPCR

2.12.5.1 DNA digestion

DNA sample were digested with restriction endonucleases at a concentration of 1 µg DNA/40 µl reaction using the vendor's (New England BioLabs Inc.) recommended conditions except that 5 U of enzyme was added per 1 µg DNA unless otherwise indicated (Table 2.11). Restriction digests were diluted a minimum of 7.5-fold in the ddPCR reaction mix to obtain the desired final DNA concentration. When higher DNA concentrations (>1 copy per droplet, CPD) were used in the ddPCR reaction (for example, positive control DNA *pinQ*), DNA was digested at the concentration of 1 µg/20 µl per reaction. After restriction digestion, the reaction were incubated at 65ºC or 80 ºC for 20 minutes (refer to Table 2.11) to deactivate the enzyme without being treated at a higher temperature to avoid denaturing the DNA target fragments.

Table 2.11. Restriction enzymes for CNV ddPCR

Restriction enzyme	*Sequence*	*Digestion Buffer*	*Incubation Temperature, ºC*	*Applied DNA sequence*
BmrI	ACTGGGNNNN/N	Cutting Smarter	37	*cysG*
BtgI	C/CRYG/G	Cutting Smarter	37	*cysG* *pinQ*
NspI	R/CATG/Y	Cutting Smarter	37	*ampC* *ampD* *ampE* *ampG*

2.12.5.2 ddPCR experiments

Genomic DNA samples were added to the Bio-Rad 2x ddPCR supermix at amounts of 0.05 ng DNA per 22 µl ddPCR reaction. Probes were present at final concentration of 250 nM (Table 2.12). Reaction mixes were briefly mixed by vortexing while avoiding the formation of bubbles, microcentrifuged for 20 seconds, then kept on ice until droplet generation.

Table 2.11. ddPCR reaction mix

Component	*1 Rxn (µl)*	*10 Rxn (µl)*
Supermix	11	110
Probe I	0.11	1.1
Probe II	0.11	1.11
Primer I-F	0.9	9
Primer I-R	0.9	9
Primer II-F	0.9	9
Primer II-R	0.9	9
Restriction enzymes	0.2 (0)	2 (0)
Water	2.98 (3.18)	29.8 (31.8)
DNA (1:500)	4	4
Total	22	220

Sample were converted into droplets, eight sample at a time, by adding 20 µl of sample to each sample well of the DG8^{TM} cartridge followed by addition of 70 µl of droplet generation oil to each of the corresponding oil wells. The cartridge was then placed into the QX200 droplet generator within 2 minutes after oil addition for droplet generation. Droplets were transferred from the droplet wells in the cartridge with an 8-channel P50 pipette to 96-well PCR plate. As many as 12 sets of samples were successively prepared in cartridges and transferred to the PCR plate before it was heat sealed with a pierceable foil seal. The plate was transferred to a thermal cycler and, uncles otherwise specified, reactions were run under the following standard cycling conditions (Table 2.13).

Table 2.12. ddPCR reaction cycles

Temperature	*Time (minutes)*	*Cycles*
95ºC	10	
94ºC	0.5	
60 ºC	1	
		39 cycles
98ºC	10	
4ºC	∞	

After the PCR, the plate was loaded onto the QX200 Droplet Digital reader, which automatically reads the droplets from each well of the plate (~36 wells/hour). Data analysis was performed using Quantasoft^{TM} software (Bio-rad). Negative droplets, lacking target and/or reference gene DNA, and positive droplets, containing multiple copies of DNAs (used gene *pinQ* in this study), were counted to give the fraction of positive droplets.

2.12.5 Copy number variations (CNV) calculations

CNV analysis by ddPCR involves quantification of target and reference loci through the use of duplex target and reference assays. In QuantaSoft^{TM} software, copy number is determined by calculating the ratio of the target DNA concentration to the reference DNA concentration, times the number of copies of reference species in the genome (Equation 2.2). The error bars on a CN estimate in QuantaSoftTM software are the 95% confidence interval of this measurement.

$CN = \frac{A}{B} N_B$ *(Equation 2.2)*

A = concentration of target species

B = concentration of reference species

N_B *= number of copies of reference loci in the genome*

2.12.6 mRNA levels analysis used by ddPCR

mRNA levels analysis follows the same workflow as the DNA copy number analysis used by ddPCR (Chapter 2.12.5) with the benefit that allows directly partition sample RNA

instead of DNA. The sample is partitioned into 20,000 droplets, with target and background RNA randomly distributed among the droplets. After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target RNA was present or not present after partitioning. Each droplet provides an independent digital measurement. Positive and negative droplets are counted, and software calculates the concentration of target RNA as copies/µl.

2.13 Whole genome sequencing

Genomic sequencing is explored following the Nextera Flex library preparation kit process (Illumina, USA) with adaptation. Briefly, genomic DNA was quantitatively assessed using Quant-iT picogreen dsDNA assay kit (Invitrogen, USA). The sample was normalised to the concentration of 1 ng/µl. 10 ng of DNA was used for library preparation. After tagmentation, the tagmented DNA was amplified using the facility's custom designed i7 or i5 barcodes, with 12 cycles of PCR. The quality control for the samples was done by sequencing a pool of samples using MiSeq V2 nano kit – 300 cycles. Briefly, after library amplification, 3 µl of each library was pooled into a library pool. The pool is then clean up using SPRI beads following the Nextera Flex clean up and size selection protocol. The pool was then sequenced using MiSeq V2 nano kit (Illumina, USA). Based on the sequencing data generated, the read count for each sample was used to identify the failed libraries (*i.e.* libraries with less than 100 reads). Moreover, based on the read count, libraries were pooled at a different amount to ensure equal representation in the final pool. The final pool was sequenced on Illumina NovaSeq 6000 Xp S4 lane, 2 × 150 bp at Ramaciotti Centre for Genomics (University of New South Wales, Australia).

2.14 Stochastic Optical Reconstruction Microscopy (STORM)

STORM was used for the detection of the various protein in *E. coli* strains, under various growth conditions, specified in the text below. The STORM method used in this study is based on the method previously described in [128] with variations.

2.14.1 Chemicals and regents for immunofluorescence

The chemicals, regents and antibodies used in STORM for immunofluorescence are all listed below:

Table 2.13. Cell extraction solution (pH 7.0)

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
PIPES	1 M (10 ×)	0.1 M	5 ml	*TritonX-100 needs to be added freshly before the extraction*
EGTA	0.1 M (100 ×)	1 mM	0.5 ml	
$MgCl_2$	1 M (1000 ×)	1 mM	50 µl	
TritonX-100	10% (50 ×)	0.2%		
ddH_2o			Up to 50 ml	
Total			50 ml	

** Total 200 -500 µl solution for each culture well.*

*** Exaction time ranges from 60 ~70 seconds.*

Table 2.14. Cell fixation solution

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
BSA	10% (2 ×)	5%	500 µl	*TritonX-100 needs to be added freshly before the fixation*
TritonX-100	10% (20 ×)	0.5%	50 µl	
PBS	1 ×		450 µl	
Total			1000 µl	

Table 2.15. Primary-antibody solution

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
Antibody			2 ~10 µg/ml	*Concentration of antibody needs to be optimized before use*
BSA	10% (2 ×)	5%	100 µl	
TritonX-100	10% (20 ×)	0.5%	10 µl	
PBS	1 ×		90 µl	
Total			200 µl	

** Concentration of antibody needs to be optimized before use.*

Table 2.16. Secondary-antibody solution

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
Antibody			2 ~3 µg/ml	*Concentration of antibody needs to be optimized before use*
BSA	10% (2 ×)	5%	100 µl	
TritonX-100	10% (20 ×)	0.5%	10 µl	
PBS	1 ×		90 µl	
Total			200 µl	

** Concentration of antibody needs to be optimized before use.*

Table 2.17. Antibody list

Antibody	*Purchased from*
RecA	Abcam, ab63797
DNA Pol II	Abcam, ab188424
RNA Pol II	Abcam, ab191598
FtsZ	Agrisera, AS07217
Secondary Antibody	Abcam, ab150075

Table 2.18. EdU stock solution (1000 ×)

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
EdU	Powder 5mg	10 mM		*Recover to room temperature before use*
DMSO			2 ml	
Total			2 ml	

Table 2.19. 1 × Chick-iT EdU solution

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
EdU reaction Component-D	10 ×	1 ×	4 ml	*Recover to room temperature before use*
ddH_2O			36 ml	
Total			40 ml	

** 900 μl per tube stored at 4℃.*

Table 2.20. 10 × Chick-iT EdU solution

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
EdU reaction Component-F	Powder	1 **vial**		*Recover to room temperature before use*
ddH$_2$O			2 ml	
Total			2 ml	

** 200 µl per tube stored at -20℃.*

Table 2.21. Chick-iT reaction blocking buffer

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
BSA	10% (2 ×)	5%	500 µl	*Recover to room temperature before use*
TritonX-100	10% (20 ×)	0.5%	50 µl	
PBS	1 ×		450 µl	
Total			1 ml	

** 3 ~5ml buffer was used for each culture well.*

2.14.2 Chemicals and regents for STORM imaging

The chemicals, regents and antibodies used in STORM for cell imaging are all listed below:

Table 2.22. STORM imaging buffer A (pH 8.0)

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
Tris/HCl (pH 8.0)	1 M (10 ×)	100 mM	3 ml	
NaCl	5 M (250 ×)	20 mM	120 µl	
Glucose	30 % (3 ×)	10%	10 ml	
ddH$_2$O			Up to 30 ml	
Total			30 ml	

** filtered by 0.22 µm filter and stored on vacuum.*

Table 2.23. STORM imaging buffer B (pH 8.0 ~8.5)

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
PBS (pH 7.4)	1 M (10 ×)	100 mM	10 ml	
NaOH	5 M	0.5%	50 µl	
ddH$_2$O			Up to 100 ml	
Total			100 ml	

** filtered by 0.22 µm filter and stored on vacuum.*

Table 2.24. Buffer C for glucose oxidase & catalase solution

Composition	*Stock concentration*	*Working concentration*	*Volume*	*Information*
Tris/HCl (pH 8.0)	1 M (100 ×)	10 mM	300 µl	
NaCl	5 M (100 ×)	50 mM	300 µl	
ddH$_2$O			Up to 30 ml	
Total			30 ml	

** Refer to Nikon^{TM} production manual*

Table 2.25. Glucose oxidase & catalase solution (GLOX)

Composition	*Stock concentration*	*Working concentration*	*Volume/Amounts*	*Information*
Glucose oxidase	Powder	60 mg/ml	120 mg	
Catalase	Powder	6 mg/ml	12 mg	
Catalase	20 mg/ml (4 ×)	6 mg/ml	500 µl	
Glycerol	80% (2 ×)	40%	Up to 1 ml	
Buffer C			Up to 2 ml	
Total			2 ml	

** Centrifuge at 200,000 g for 1 ~2 minutes.*

*** 200 µl per tube stored at -20ºC for at most 1 month.*

Table 2.26. Trolox vitamin E solution

Composition	*Stock concentration*	*Working concentration*	*Volume/Amounts*	*Information*
Trolox	Powder	100 mM		
DMSO			Up to 1 ml	
Total			1 ml	

** Stored at room temperature.*

Table 2.27. STORM imaging solution A

Composition	*Stock concentration*	*Working concentration*	*Volume/Amounts*	*Information*
Buffer A	1 ×		Up to 2 ml	
β-ME	14.4 M (100 ×)	144 mM	20 ~30 µl	
MEA	1 M (10 ×)	0.1 M	200 µl	
GLOX	100 ×	1 ×	20 µl	
Total			2 ml	

** Fresh prepared at room temperature before use.*

Table 2.27. STORM imaging solution B

Composition	*Stock concentration*	*Working concentration*	*Volume/Amounts*	*Information*
Buffer A	1 ×		1.96 ml	
β-ME	14.4 M (100 ×)	144 mM	20 µl	
Trolox	1 mM (100 ×)	1 ×	20 µl	
GLOX	100 ×	1 ×	20 µl	
Total			2 ml	

** Fresh prepared at room temperature before use.*

2.14.3 Software and codes for STORM imaging

Software and codes used in this study for STORM imaging are all listed below:

Table 2.28 Software and codes for STORM imaging

Software	*From*
NIS Elements Advanced Research	Nikon, Japan
Meta-Morph	Molecular Device, US
LabView	National Instrument, US
Fiji/ImageJ	Image Processing and analysis in Java http://rsb.info.nih.gov/ij/index.html，US
Insight3	Dr. Bo Huang, UCS, US
Metlab	Mathworks, US
Photoshop CS	Adobe, US
Vaa3D	Hanchuan PENG Lab, US http://home.penglab.com/proj/vaa3d/Vaa3D/About_Vaa3D.html
Fi3sta	Dr. Felix Ruhnow, UPen, US
Hal4000	Dr. Hazen Babcock, HU, US

2.14.4 STORM imaging

2.14.4.1 Dish washing

A 35 mm cell culture dish (0.17 mm) was placed in a large beaker (keep the dish upright and straight) for the rinsing once with the ultrapure water MilliQ. 1 M KOH was added into the beaker that is then placed in an ultrasonic cleaning instrument for washing 30 minutes. Using MilliQ water to rinse the culture dish for three times. Then, the dish was placed back to the ultrasonic cleaning instrument for the secondary washing following with the same protocol. After the washing, ultrapure water was discarded and the dish was dry using high-purity nitrogen and UV exposure for 30 minutes. Dishes were stored in a cool and dry place until the use.

2.14.4.2 DNA labelling using EdU

An overnight culture was resuspended into a fresh LB medium for growing to the logarithmic phase with OD_{600} = 0.4 -0.8. This culture was then added into 10 μM EdU (final concentration) (Thermofisher, USA) and cultured at 37ºC for overnight with shaking at 250 rpm. The toxicity of EdU was detected through the bacterial growth monitoring (Fig. 2.3).

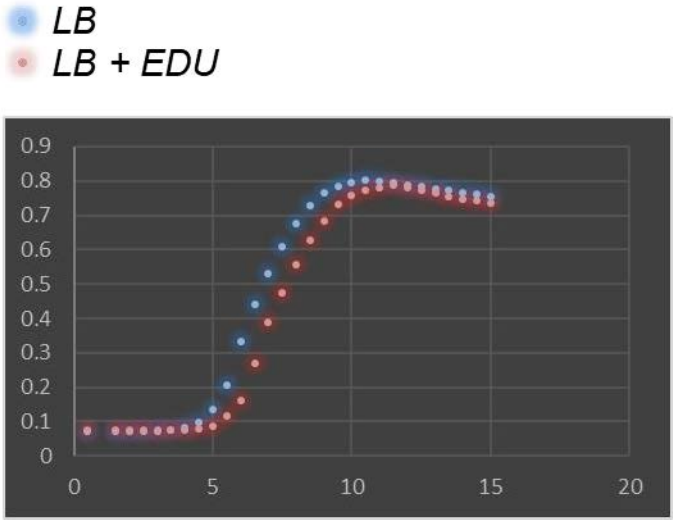


Figure 2.3 Addition of EdU did not influence the growth of bacterial cells. *Bacterial cells were cultured in the absence or presence of EdU at 10 μM for overnight at 37ºC with shaking at 250 rpm. The growth was monitored by an automated plate reader to reflect the toxicity of addition of EdU. The OD value was plotted to display the bacterial growth curve.*

2.14.4.3 Fixation of cells

Cells were fixed with $NaPO_4$ (30 nM), formaldehyde (2.4%), glutaraldehyde (0.04%). Samples were incubated at room temperature for 15 minutes followed by 45 minutes on ice. Samples were then centrifuged to pellet cells and the supernatant discarded. Cell pellets were washed twice with phosphate-buffered saline (PBS), pH 7.4. Cells were then resuspended in 200 μL of GTE buffer. Samples were kept on ice until the next step.

2.14.4.4 Immunostaining

Wells were rehydrated by adding PBS and incubating at room temperature for 4 minutes. The PBS was then aspirated. Each well was then incubated with PBS containing 2% w/v BSA (BSA-PBS) for 15 minutes at room temperature in a humidity chamber. The humidity chamber was constructed by adding wet tissue paper to a petri dish. The solution was then aspirated and 20 μL of the primary antibody in a 1 in 10,000 dilution in BSA-PBS, was added to each well. This was then allowed to incubate overnight in a humidity chamber at 4oC. The following morning, the primary antibody was aspirated off and each well was washed 10 times with PBS to remove any unbound antibody. A volume of 20 μL secondary antibody diluted 1 in 10,000 in BSA-PBS was added to each well. The slide was incubated for 2 hours in the dark, at room temperature in the humidity chamber. Wells were washed 10 times with PBS to remove excess secondary antibody. An imaging buffer (100 mM Tris/HCl pH 8.0, 20 mM NaCl and 10% glucose, all purchased from Sigma-Aldrich) and an oxygen scavenger system (60 mg/ml Glucose oxidase and 6 mg/ml catalase, both purchased from Sigma-Aldrich) were used for the STORM imaging. 140 mM β-mercaptoethanol was added to promote photo-switching.

2.14.4.5 Image analysis

STORM image analysis, drift correction, image rendering, protein cluster identification and images presentation were performed using Insight3 (gift of Prof. Bo Huang from UCSF), custom-written Matlab (2012a, MathWorks) codes, SR-Tesseler (IINS, Interdisciplinary Institute for Neuroscience), and Image J.

2.15 Coulter counter analysis of cell length

A Coulter counter (Beckman) measures the size of particles, such as bacterial cells, suspended within an electrolyte buffer. This size is reported as a volume (μm^3), and in the case of rod-shaped bacteria such as *E. coli* is proportional to the cell length. A volume of 100 µL of fixed cells was added to 9.9 mL of Isoflow buffer (Beckman). Of this, 200 µL was run through a 50 µm aperture tube, and data collected over 400 bins ranging from 0.6 μm^3 to 100 μm^3. Data was exported in excel as a table of bin width and number of cells per bin.

2.15.1 Graphing coulter counter data

The coulter counter data was plotted as a histogram with the cell volume along the x-axis and percentage of cells on the y-axis, in Mathematica (Wolfram).

2.15.2 Statistical analysis of the coulter counter data

The Coulter counter counts hundreds of thousands of cells per sample run, meaning that even very small differences between populations will be statistically significant. This includes the normal low levels of variability between two samples from the same strain, and does not necessarily indicate biological significance. As such, statistical analysis was not used to determine significant differences between strains. Instead, differences were determined visually from the distribution graph and using a cross correlation function, calculated in Mathematica (Wolfram). Cross correlation gives a measure of how similar

two distributions are, with a value of 1 being identical, a value of 0 being un-correlated and -1 being anti-correlated.

Chapter 3. Single exposure of β-lactams antibiotics triggers bacterial evolution of resistance

Chapter 3. Single exposure of β-lactams antibiotics triggers bacterial evolution of resistance

3.1 Introduction

Antibiotics can induce bacterial evolution to be persistent, tolerant and even resistant which uncovers many molecular mechanisms, including genetic mutations in drugs target, modification of enzyme that directly inactivates the drugs or activation of efflux pump that reduces the concentration of drugs in cells.

3.1.1 Antibiotics induced emergence of persisters

Bacterial persistent to β-lactams antibiotics has been extensively studied. For example, using a microfluidic device, Balaban *et al.* find that some persister cells are pre-existed in the subpopulation prior to exposure to antibiotics, but some others can arise from growth-inhibited bacteria within growing populations [1]. Both of these two types of persister cells can survive and successfully resume growth after antibiotic removal. Similar results are reported using additional techniques such as fluorescence activated cell sorting method in which under an experimental condition majority of bacterial persister cells (~80%) are arose from non-growing subpopulation, whereas the remainder originated from cells growing prior to treatment. In addition to the lack of bacterial growth, Pu *et al*. use *in vivo* fluorescent imaging and next-generation sequencing methods to observe that under β-lactams antibiotics treatment bacterial persisters exhibit less cytoplasmic drug accumulation as a result of enhanced efflux activity in addition to physiological quiescence, a double insurance strategy to ensure its survival under

antibiotic attack [2]. Further, they demonstrate that each survived persister cell has different “dormancy” depth, which in turn regulates whether and when it can resume growth after antibiotic removal. In particular, they find that persister cells are in shallow dormancy depth, whereas the viable but non-culturable cells are in deep dormancy depth, which is modulated by endogenous protein aggregation [3].

In addition to β-lactams antibiotics, several other kinds of antibiotics are reported to induce the formation of bacterial persister. For example, Wakamoto *et al.* use time-lapse microscopy to investigate the formation of persistence to isoniazid in mycobacteria and find that phenotype represents a dynamic equilibrium between cell growth and stochastic expression of the prodrug activating enzyme *katG* [4]. Fluoroquinolones kill bacterial cells via the corruption of DNA topoisomerases, including DNA gyrases, which have a vital role in replication and transcription [4]. It is thereby believed to induce bacterial persistence regardless of their growth status. For instance, Dorr *et al*. demonstrated that pretreatment of *E. coli* with low concentrations of ciprofloxacin can trigger the formation of persister cells to higher level of ciprofloxacin from stationary subpopulation. Meanwhile, these persister cells are mostly induced in a functional SOS response manner [5].

Kester and Fortune first defined the term of tolerance that enables bacterial cells to survive a transit exposure to antibiotics at lethal concentration [6]. In contrast to persistence which is the ability of a subpopulation of a clonal bacterial population to survive exposure to high concentration of antibiotics, tolerance may be acquired through a genetic mutation or conferred by environmental conditions [7]; for example, several classes of antibiotics are reported to induce formation of tolerance under poor bacterial

growth conditions. Lederberg *et al*. report that in the absence of essential amino acid auxotrophs mutant isolates can survive as tolerant cells when is exposed to lethal concentration of penicillin [8].

3.1.2 Experimental evolution of resistance

Antibiotics induced bacterial resistance is typically caused by inherited *de novo* mutations, which is associated with numerous molecular mechanisms that have been reviewed in Chapter I. It is of great importance to note that tolerance can facility the evolution of resistance under experimental condition. Many discoveries show that cellular activity and division can occur during the formation of persistence raises the possibility that persisters function as an intermediate state in the elaboration of heritable drug resistance [9]. Residual cell division combined with active survival mechanisms could support or even enhance the development of heritable genetic changes, because many of the same stress-response programs important for the generation and survival of persisters can also accelerate genome-wide mutagenesis and horizontal gene transfer, which is regarded to represent a pool of adaptively evolving organisms from which resistant mutants can emerge [9] (Fig. 3.1). For example, Irit *et al*. use cyclic treatment of ampicillin (50 μg/ml) to kill *E. coli* cells and observe an increase in MIC after 7 to 17 cycles, but more interestingly most bacterial cells show delayed growth after 3 to 4 cycles when plated on fresh medium indicating the formation of tolerance. By exploring the genome they find that tolerant strains bear tolerance mutations which are present prior the appearance of resistance mutations, and the tolerance mutations are able to cause the establishment of resistance mutations in the population. These findings first demonstrate that tolerance

plays a crucial role in the evolution of resistance in a bacterial population under exposures to lethal concentration of antibiotics.

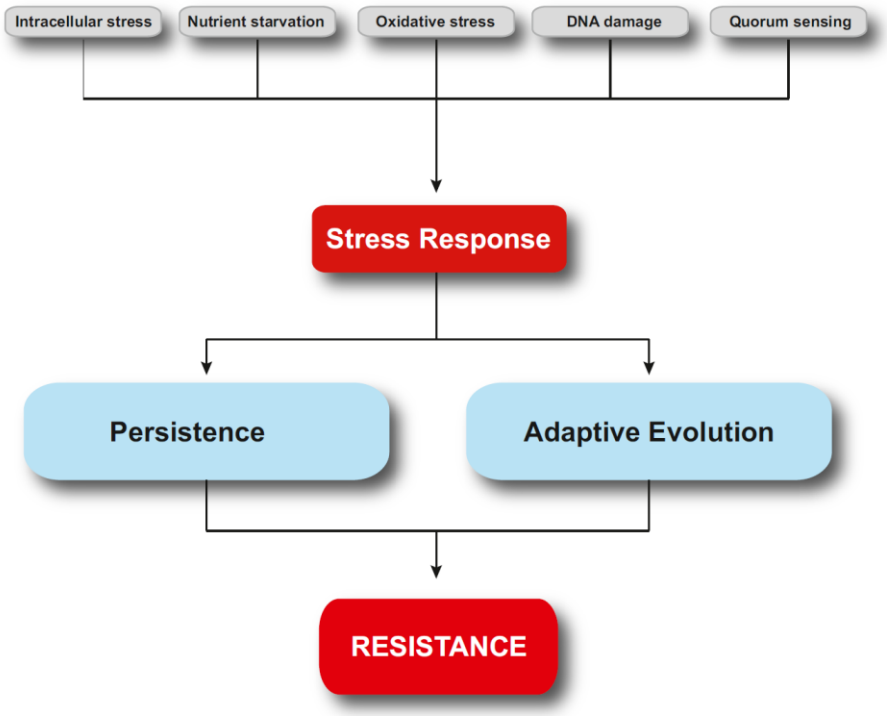


Figure 3.1 Stress responses link persistence, adaptive evolution and resistance. *Microbial responses to endogenous or exogenous stresses promote survival as well as genetic plasticity. Persistent organisms undergo rapid adaptive evolution and can function as a reservoir for the elaboration of drug resistance [9].*

Although bacterial cells can be triggered to be resistant by antibiotics exposure, the variables that influence the rate of emergence of resistance are not well understood. Using a microfluidic device designed to mimic naturally occurring bacterial niches, ciprofloxacin can induce the emergence of resistance of *E. coli* within 10 hours. This resistance emerged with as few as 100 bacteria in the initial inoculation. To further understand how evolution is shaped by the diversification potential and differences in

adaptive constraints of large populations in spatial environments, Michael Baym *et al*. design a large experiment device, the microbial evolution and growth arena (MEGA) plate, in which bacteria spread and evolved on a large antibiotic landscape that allows visual observation of mutation and selection in a migrating bacterial front. They find that evolution is not always resulted from the most resistant mutants; highly resistant mutants may be trapped behind more sensitive lineages, even though the mechanism is not clear yet.

3.1.3 Bacterial tolerance drives the evolution of resistance

Another question remaining unclear is that what factors determine the evolution of resistance from persistence or tolerance. A recent study reports that a single exposure of fluoroquinolone antibiotics can induce impressive SOS responses and the formation of persistence. Furthermore, many of the resulting daughter cells continue to replicate and resume normal appearance with an increased resistant mutation frequency. This resistance is heritable, enriched in persister-derived populations, and significantly dependent on the activation of RecA.

The function of RecA has been introduced and discussed in Chapter I. During the emergence of resistance, RecA turns to be regarded as an indicator to accelerate the evolution of resistance from tolerance. For example, a point mutation in the Staphylococcus aureus *pbpB* gene coding for penicillin-binding protein 2 caused decreased susceptibility to β-lactams antibiotics which is regulated through the activation of RecA on SOS responses [10]. In a recent study, mutations occurring on genes *gyrA* and *parE*, encoding DNA gyrase and DNA topoisomerase IV are found in an antibiotic

resistant *Pseudomonas aeruginosa* strain [11]. Further, deletion of *recA* in *E. coli* and *Staphylococcus aureus* can lead to a significant antibiotic induced resistance reduction [12]. Horizontal transfer of drug-resistance genes is also mediated by the activation of RecA which plays a central role in the acquisition and dissemination of antibiotic resistance genes among bacteria cells. For example, SOS responses significantly stimulates the transfer of integrating genetic elements which contains genes conferring resistance [13]. Suppression of RecA activation is thereby proposed as a possible therapeutic strategy to suppress the development of antibiotic resistance. A proposed RecA inhibitors shows the property that it can block the fluoroquinolone induced activation of SOS responses, and in turn reduce the acquisition of antibiotic resistance mutations and horizontal transfer of mobile genetic elements [14].

However, it is not yet well-known that what the role of RecA is in the emergence of resistance induced by β-lactam antibiotics. This chapter therefore aims to investigate the function of RecA during the evolution of resistance.

3.1.4 Chapter aims and objectives

The overall aim of this chapter was to understand the role of *recA* in the emergence of resistance induced by exposure of β-lactams antibiotics. In particular, the first aim was to investigate if the exposure of ampicillin triggered the evolution of tolerance or resistance in wild type and *ΔrecA* strains.

The second aim was to find what caused the evolution of tolerance and resistance to β-lactams antibiotics. These objectives were achieved by a batch culture experiment in

which bacterial cells were treated by a single exposure of ampicillin at lethal concentration.

3.2 Results

3.2.1 Single exposure of ampicillin triggers formation of tolerance in wild type *E. coli* strain

I exposed batch cultures of the wild type bacterium *E. coli* MG1655 to a high concentration of ampicillin at 50 μg/ml which is 10 times greater than the MIC but comparable to therapeutic doses for 0, 4, and 8 hours respectively (Fig. 3.2). After the incubation, the ampicillin-containing medium was removed by washing twice. Half of the cultures were directly analysed and another half were resuspended in fresh medium for secondary overnight culture.

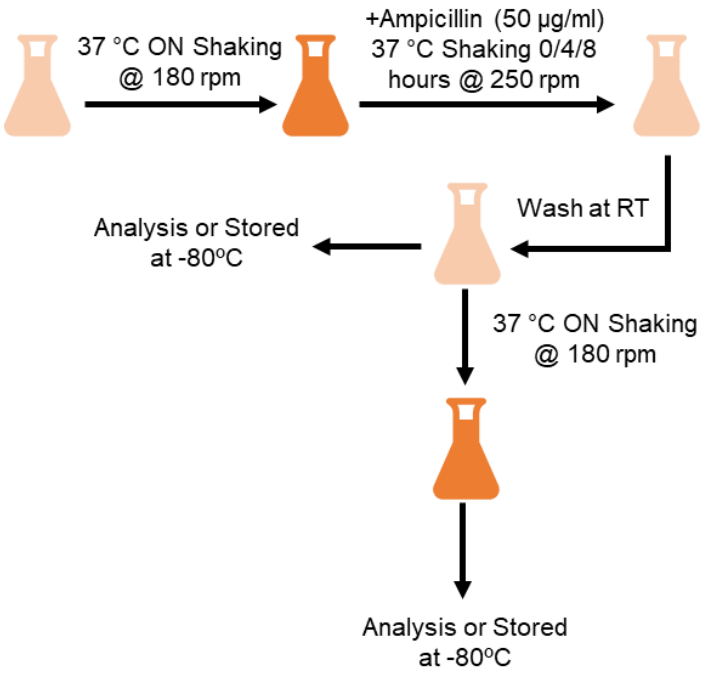


Figure 3.2 Evolutionary protocol of antibiotic exposure. *The experimental evolution protocol consists of three steps: 1) Killing. An overnight bacterial culture (~10^9 counts/ml) is diluted 1:100 into 50 ml LB medium with an addition of ampicillin at 50 µg/ml for 0m 4, and 8 hours incubation at 37 ºC with shaking at 250 rpm. 2) Washing. LB medium containing antibiotics are removed by washing twice in LB medium (centrifugation at 1,500×g for 20 min). In order to avoid cycle-to cycle fluctuations in the residual amounts of antibiotics, I add selected antibiotics at 1/100 of the high concentration used before in to the washing medium, above the uncertainly due to the washing procedure. 3) Growth. Half of the bacteria is directly analysed or stored at -80 ºC. Another half of culture is resuspended in 50 ml fresh LB medium and grown overnight for 18 hours at 37 ºC with shaking at 180 rpm. Regrown culture is then used for analysis or stored at -80 ºC.*

I first found that after 4 and 8 hours killing, both of the proportion of surviving bacteria were less than 0.01% which proves that wild type *E. coli* was sensitive to ampicillin

exposure (Fig. 3.3A). The increased survival rate of the evolved bacteria could have been achieved by a mutation that conferred resistance. However, I found that the MIC of ampicillin for clones isolated from 4 and 8 hours treated bacterial evolved lines was indistinguishable from that of untreated cell lines (Fig. 3.3B). I next tested the MDK_{99} (the minimum duration for killing 99% of cells) which can be defined to quantify tolerance, as a higher tolerance translates to a longer MDK_{99} , in other words a treatment of longer duration is need to reach the same level of killing for tolerance. It is interesting that I found the MDK_{99} of the evolved population increased with the duration of the stress period, reaching values as high as 15 times the MDK_{99} of the untreated cells (Fig. 3.3C). I therefore conclude that these populations have all adapted to the antibiotic regimen through tolerance not resistance.

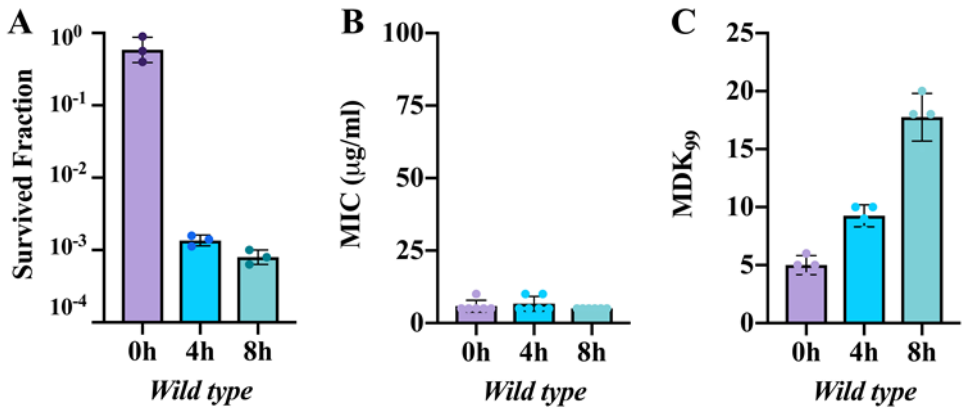


Figure 3.3 Bacterial evolution of tolerance induced by a single exposure of ampicillin. *(A) Survival of the bacterial cells after the killing of ampicillin at 50 μg/ml for 4 and 8 hours incubation. (B) The MIC of survived bacterial cells relative to the untreated cells. A representative line of each killing duration is shown. MICs of the batch cultures are shown, after each killing duration. (C) Increase to tolerance of the survived bacterial cells. MDK_{99} was determined by measuring the time to kill 99% of strains as descripted above. All data are presented as the mean ± s.d. of four independent experiments.*

Different mechanisms can enable bacteria to endure an interval of exposure to antibiotics. One way to achieve tolerance is by slowed growth. Another strategy for tolerance is related to the cells being in stationary phase before exposure to antibiotics, resulting in a delay in regrowth when switched to a new environment. By extending the time to first division (the single cell lag time) and remaining longer in a dormant state, a cell may avoid the harms of antibiotics. A case in point is the tolerance of type I persistent bacteria, which is based on the existence of a subpopulation of cells with sufficiently long single-cell lag times. To explore what types of tolerance I observed in above experiment, I further utilized a ScanLag system to perform a phenotypic characterization that enables the measurement of the distribution of the lag period and growth of single colonies using

an automated scanner setup (Fig. 3.4A and B). I found that bacterial cells after 4 and 8 hours ampicillin exposures showed delayed appearance time for around 7 hours when plated all on fresh medium (Fig. 3.4C), which was caused by an extended lag time that conferred tolerance to the ampicillin with a regular growing rate (Fig. 3.4D). I therefore conclude that these survived cells have all adapted to the antibiotic regimen through tolerance by lag-phase but not resistance.

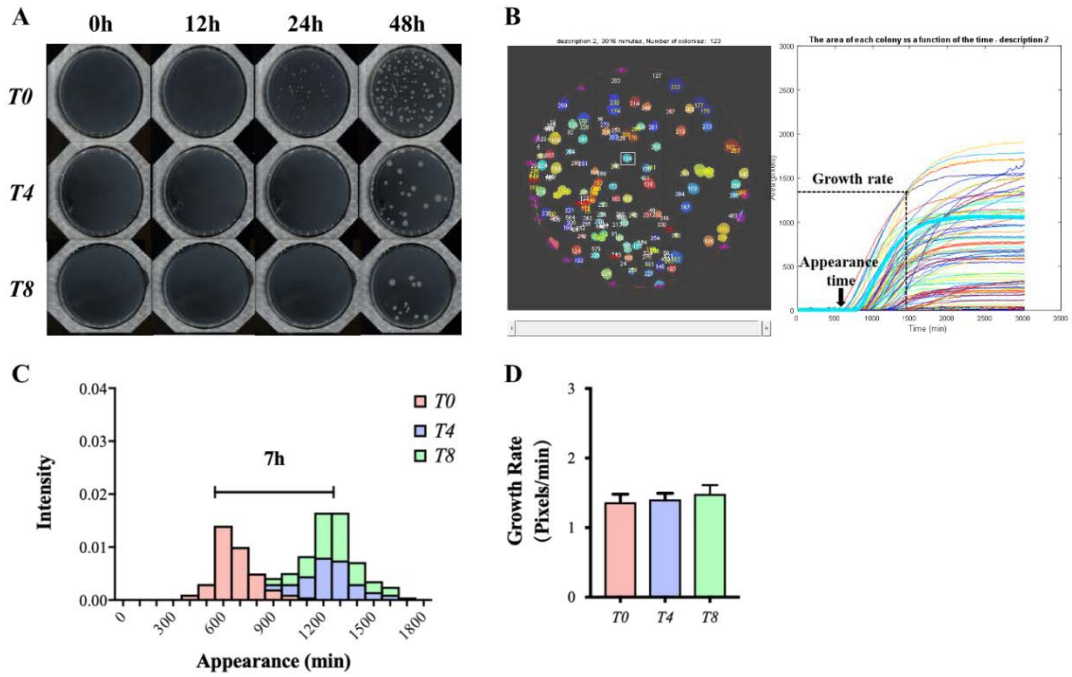


Figure 3.4 Monitoring the population dynamics of tolerance. *(A and B) Detection of colony appearance and growth time using Scanlag on the evolved tolerant cells. (C)The histograms show the proportion of colony-forming units (CFUs) detected at each time point from 0 to 48 hours incubation, with s.e.m. below 8% and sample size of n=124 (T0), n=89 (T4), and n=77 (T8), respectively. (D) Colonies appearing at later times grow at the same growth rate, indicating an emergence of tolerance with lag-phase but not by growth-arrested.*

3.2.2 Deficiency of *recA* promotes bacterial evolution of resistance

To test the role of *recA* in the emergence of resistance induced by β-lactam antibiotics, I exposed batch cultures of several *E. coli* strains with genetic modification to a single exposure of ampicillin at 50 μg/ml (10×MIC) for 0, 4, and 8 hours. I first found that ampicillin was not as effective at killing the *ΔrecA* strain compared with that to wild type cells because the number of survived cells was increased from 10^{-3} to 10^{-2} (CFU) after 4 hours treatment, and 10^{-3} to 10^{-1} (CFU) after 8 hours treatment, respectively (Fig. 3.5A). However, complementing the *ΔrecA* strain with a plasmid contacting the *recA* expression store and overexpression of *recA* in wild-type strain could resume bacterial cells sensitive to ampicillin exposure (Fig. 3.5A).

To compare the resistance capacity of survived cells to ampicillin, I measured the MIC value and found that it was indistinguishable in wild-type strain but could reach to 20-fold greater in *ΔrecA* strain (Fig. 3.5B). It is interesting that complementing the *ΔrecA* strain with a plasmid contacting the *recA* expression restore could sensitize the *ΔrecA* strain to ampicillin; however, overexpression of *recA* by plasmid transformation had no effects on the emergence of resistance (Fig. 3.5B).

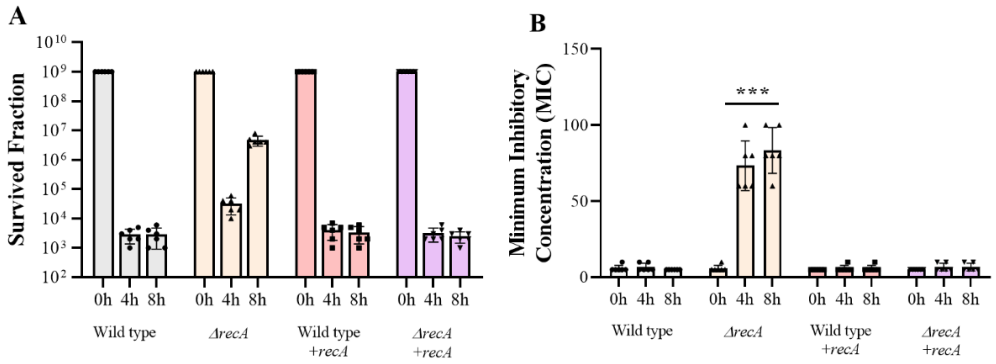


Figure 3.5 Resistance of the evolved strains. *(A) Survival of the evolved strains and ancestral strain after a single exposure of ampicillin (50 μg/ml) for 4 and 8 hours treatment. (B) Increase in resistance of the evolved strains. MIC was determined by measuring the minimum inhibitory concentration of ampicillin. All data are presented as the mean ± s.e.m. from four individual experiments. All data are presented as the mean ± s.d. of six independent experiments. *p < 0.05; **p < 0.01; ***p < 0.00. Mann-Whitney rank sum test.*

3.2.3 Frequency of genetic mutation is increased in *ΔrecA* strain

I next calculated the spontaneous mutation frequency in *E.coli* strains with and without the expression of *recA*. To identify mutations not induced by stress, I performed these measurements in the absence of antibiotic. I plated mid-exponential phase cultures on LB agar with and without ampicillin and calculated the mutation frequency by dividing the number of CFU per milliliter on ampicillin plates by the number of CFU per milliliter on LB plates (Fig. 3.6A). I compared the wild-type *E. coli* strains with and without expression of *recA*, and found that the wild-type strain had a significantly lower mutation frequency than the stains with *recA* deletion. These mutation frequencies correspond to

mutation rates of 10^{-9} and 10^{-7} mutations per generation in the wild-type and *ΔrecA* strains, respectively, providing resistance without incurring a major fitness cost. Similarly, overexpression of *recA* did not change the mutation frequency, but complementing the *recA* strain restored and further decreased the mutation frequency over that of the wild type strain (Fig. 3.6B).

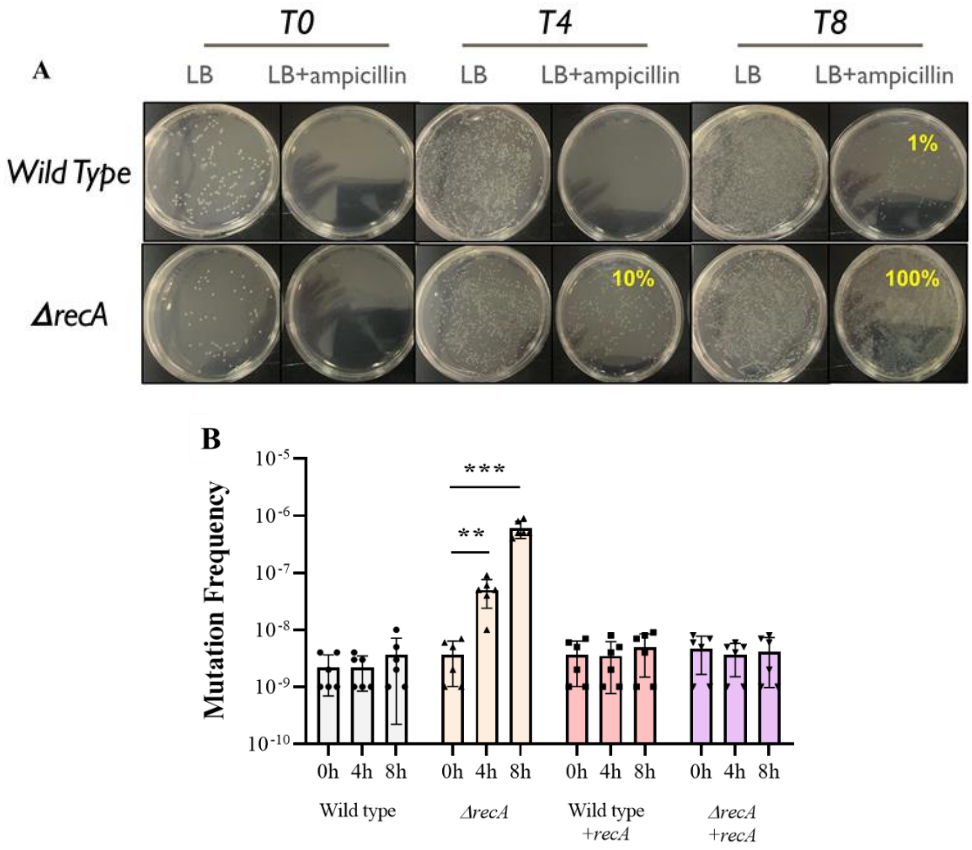


Figure 3.6 recA deletion increases the spontaneous mutation frequency. *(A) Experimental design showing an increase in spontaneous mutation in cells with or without expression of recA. (B) Ampicillin mutation frequency in E. coli wild-type, ΔrecA, recA overexpression, and ΔrecA with recA restoring strains. All data are presented as the mean ± s.e.m. from four individual experiments. All data are presented as the mean ± s.d. of six independent experiments. *p < 0.05; **p < 0.01; ***p < 0.00. Mann-Whitney rank sum test.*

3.2.4 Level of β-lactamase is dramatically elevated in *ΔrecA* strain

β-lactamase provides antibiotic resistance by breaking the β-lactam antibiotics' structure. These antibiotic all have a common element in their molecular structure: a four-atom ring

known as β-lactam. Through hydrolysis, the enzyme lactamase breaks the β-lactam ring open, deactivating the molecule's antibacterial properties (Fig. 3.7A). Therefore, increased expression of β-lactamase is typically regarded as one of the most important mechanism for bacterial evolution of resistance. I measured the expression of β-lactamase in culture medium of each strain after the exposures to ampicillin. Results showed that the expression of β-lactamase was significantly increased in *ΔrecA* strain, and complementing the *recA* strain could keep the expression of β-lactamase stable compared with that of wild-type strain (Fig. 3.7B). These results demonstrated that deficiency of *recA* could rapidly promote the evolution of resistance from tolerance after a single exposure of β-lactam antibiotic ampicillin, by which mechanism is associated with the higher expression of β-lactamase.

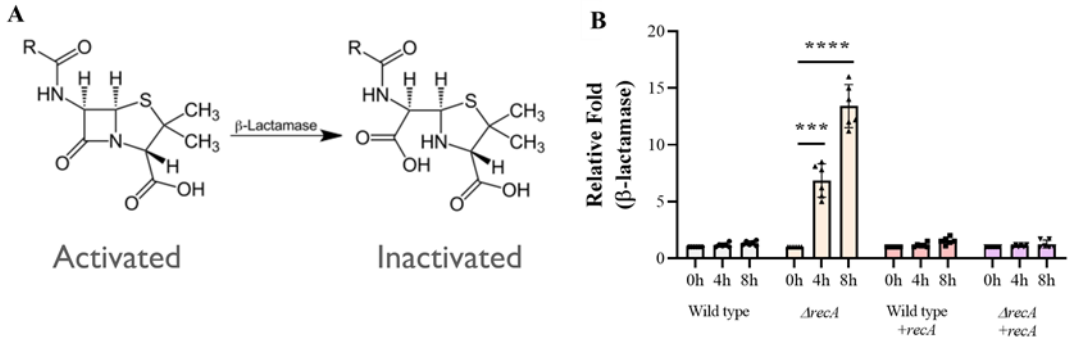


Figure 3.7 Increased level of β-lactamase in resistant isolates culture medium. *(A) Biological function of β-lactamase deactivating β-lactam antibiotics through the β-lactam ring hydrolysis. (B) Levels of β-lactamase in E. coli wild-type, ΔrecA, recA overexpression, and ΔrecA with recA restoring strains. All data are presented as the mean ± s.e.m. from four individual experiments. All data are presented as the mean ± s.d. of six independent experiments. *p < 0.05; **p < 0.01; ***p < 0.00. Mann-Whitney rank sum test.*

3.2.5 Single exposure of ampicillin induces heritable resistance in *ΔrecA* strain

To confirm if the resistance I observed in *ΔrecA* strain was heritable, I performed a bacterial passage experiment. I found that *ΔrecA* resistant isolates under 4 or 8 hours ampicillin exposure could inherit the capacity of resistance to their daughters (continuously cultured for 7 days) (Fig. 3.8A-D). I also measure the level of β-lactamases in culture medium and found that the expression of β-lactamases was highly up-regulated in all daughter cells (Fig. 3.9A-D), which may account for the reason why bacterial resistance to ampicillin was heritable.

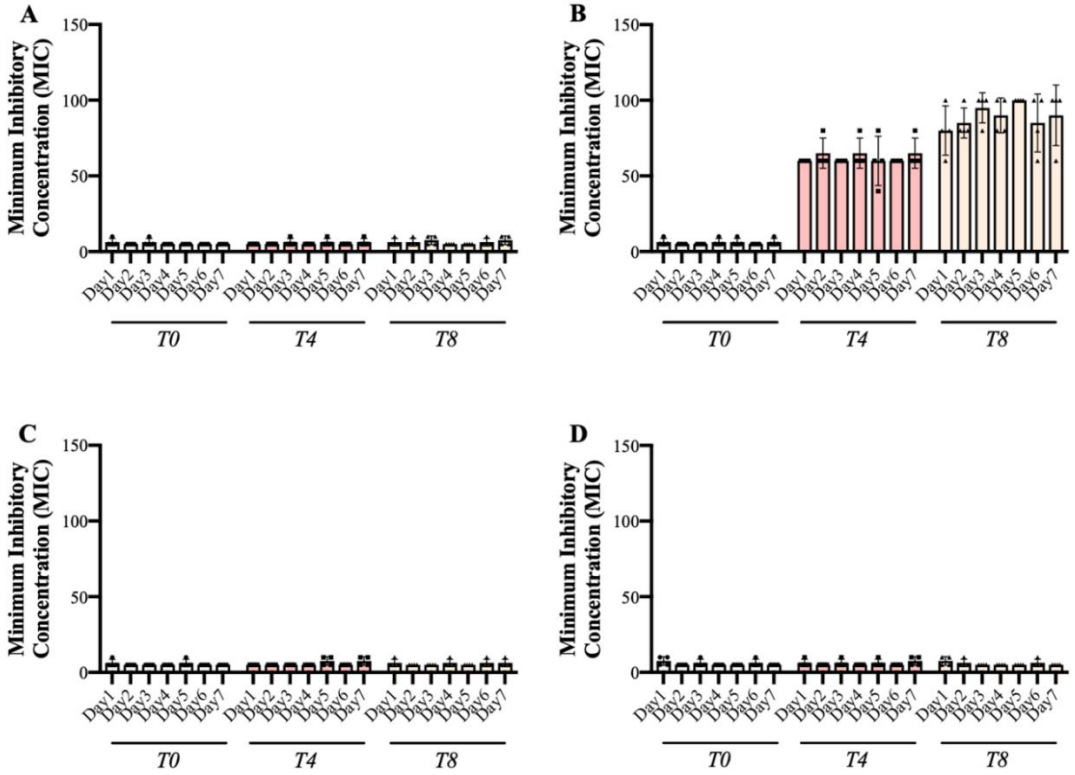


Figure 3.8 Bacterial resistance to ampicillin is heritable. *MIC value in daughter cells of (A) wild type, (B) ΔrecA, (C) Overexpression of recA, and (D) recA restoring strains. All data are presented as the mean ± s.d. of four independent experiments.*

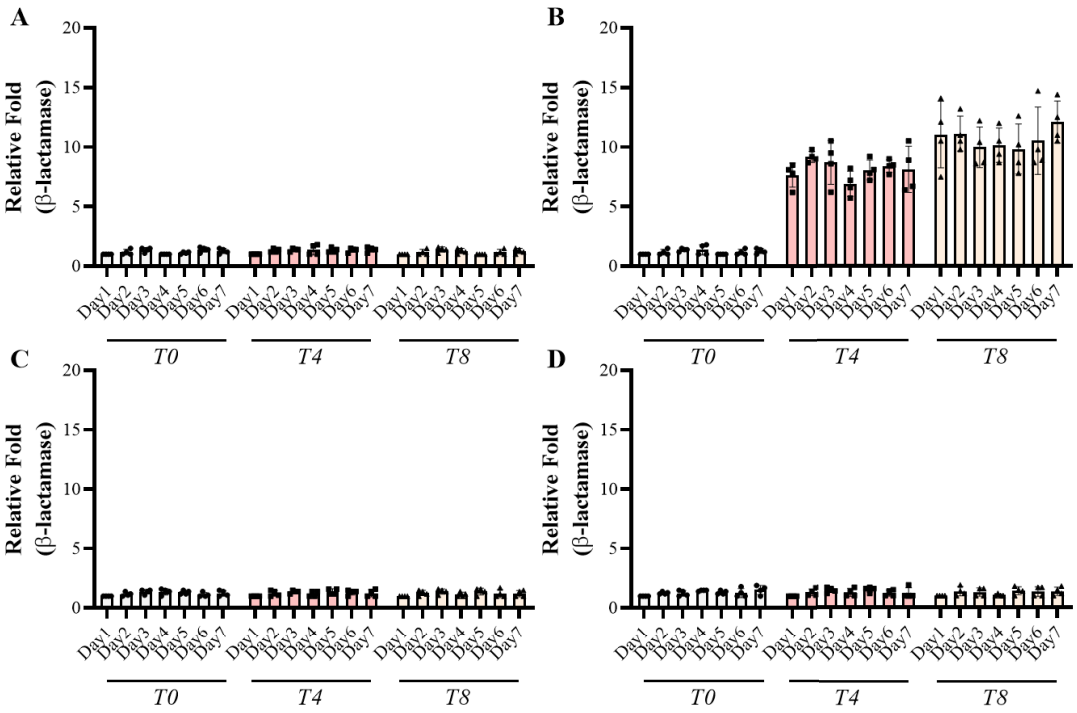


Figure 3.9 High levels of β-lactamase in daughter cells. *β-lactamase level in daughter cells of (A) wild type, (B) ΔrecA, (C) Overexpression of recA, and (D) recA restoring strains. All data are presented as the mean ± s.d. of four independent experiments.*

3.2.6 Post-*recA* restoring fails to confer drawback of bacterial resistance

It is noted that complementing the *ΔrecA* strain with a plasmid in prior to the ampicillin exposure could sensitize *ΔrecA* strain to ampicillin, I thereby hypothesized that if post-*recA* restoring was able to confer the drawback of bacterial resistance from tolerance. After 4 and 8 hours treatment with 50 μg/ml ampicillin, a plasmid contacting the *recA* expression was transferred into wild type or *ΔrecA* strain, and a blank plasmid was transferred into each strain acting as a control treatment. It was interesting that, after 9 day continuous culture, complementing the *ΔrecA* resistant isolates with post-*recA* expression restoring did not change the MIC value in *ΔrecA* strain (Fig. 3.10A).

Meanwhile, I measured the β-lactamases expression in each strain after 9 days culture, and found stable elevated expression of β-lactamase (Fig. 3.10B).

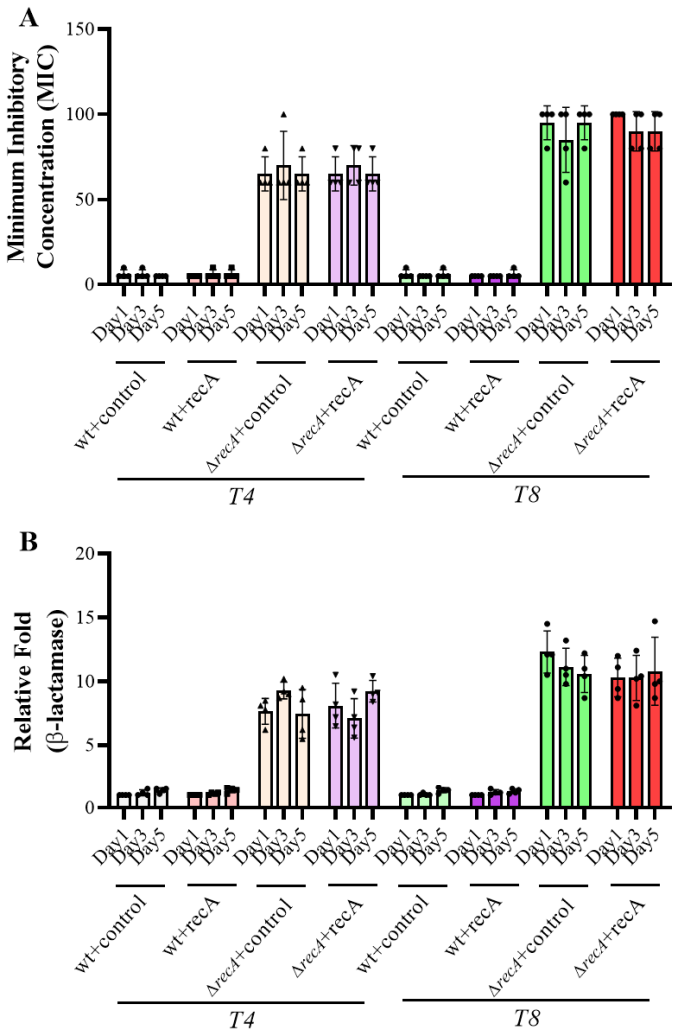


Figure 3.10 Post-recA restoring could not confer the drawback of resistance. *(A) MIC value in wild type, ΔrecA, recA overexpression, and recA restoring daughter cells transferred with a blank plasmid (control) or plasmid with the expression of recA restoring. (B) Levels of β-lactamase in wild type, ΔrecA, recA overexpression, and recA restoring daughter cells transferred with a blank plasmid (control) or plasmid with the*

expression of recA restoring. All data are presented as the mean ± s.e.m. from four individual experiments.

3.2.7 *ΔrecA* resistant isolates are not multidrug resistance

Finally, I tested if the exposure of ampicillin induced bacterial resistance in *ΔrecA* strain was multidrug resistant to other kinds of antibiotics. Untreated or 8 hours ampicillin treated wild type or *ΔrecA* strains were plated on a LB agar medium containing 5 μg/ml ampicillin, a LB agar medium containing 25 μg/ml chloramphenicol, or a LB agar medium containing 1 ng/ml ciprofloxacin, respectively. Results showed that, the exposure of ampicillin induced bacterial resistance was specifically resistant the ampicillin, and still sensitive to chloramphenicol and ciprofloxacin at 1 × MIC concentration, indicating that this *ΔrecA* resistant isolates are non-multidrug resistant (Fig. 3.11)

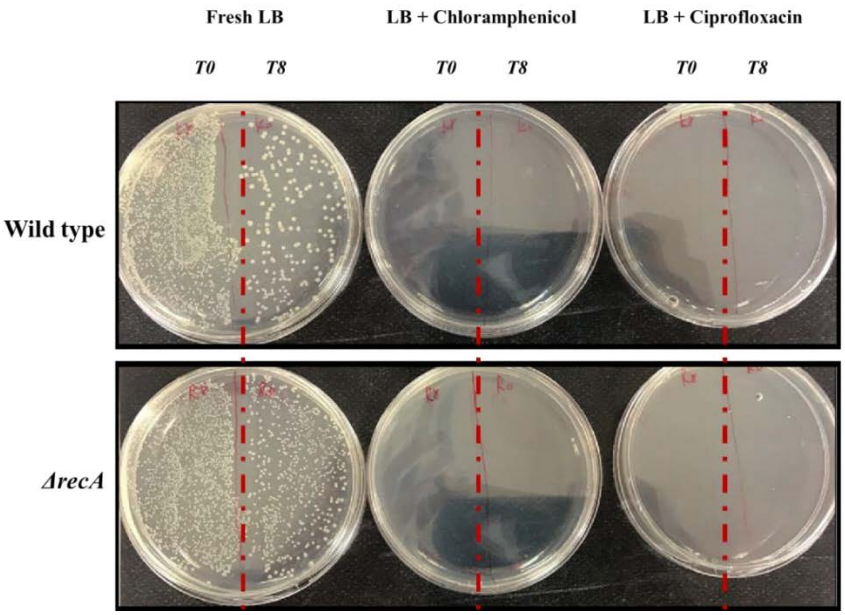


Figure 3.10 ΔrecA resistant isolates are not multidrug resistant. *ΔrecA resistant isolates could not grow on LB agar medium containing 1 × MIC chloramphenicol or ciprofloxacin.*

3.3 Conclusion

The research in this chapter was performed to initiate the investigation into what the role of gene *recA* in the bacterial evolution of resistance to antibiotics induced by the exposure of β-lactams antibiotics.

Combination antibiotic therapy is increasingly practised because of their synergistic action which can enhance their individual effects. RecA acting as a pleiotropic recombinase participates in bacterial SOS response, of which activation has been demonstrated to be required for evolution of resistance. RecA is thereby considered as a universal drug target. However, in this chapter, I believe that the development of drugs

targeting RecA is not applicable when it is designed to be used combined with β-lactams antibiotics.

In a recent paper, *recA* was demonstrated to be involved in the induction of SOS responses together with *lecA* promoting the evolution of resistance from persisters induced by ciprofloxacin. Whereas, fluoroquinolones antibiotics like ciprofloxacin can directly damage the structure of DNA, induce the DNA breaks and trigger the replication fork arrest which consequently result in the cell death. As a response, bacterial cells protect against fluoroquinolones antibiotics lethality through the induction of SOS response. However, β-lactams antibiotics do not directly disrupt the peptidoglycan turnover but can induce the SOS response through other mechanisms such as DpiBA two-component signal transduction system. The question is thereby remaining that whether β-lactams antibiotic induced resistance is relied on the activation of RecA. It is unexpected that I found deficiency of *recA* could remarkably promote the evolution of resistance within in 8 hours after a single exposure of ampicillin at lethal concentration.

I investigated what caused the emergence of resistance in the *recA* deleting strain and found in *ΔrecA* strain culture medium the expression of β-lactamase was highly increased. Since higher expression of β-lactamase contributes to the resistance to β-lactams antibiotics, the treatment of ampicillin resulted in a broad resistance formation to other β-lactams antibiotics including penicillin G and carbenicillin.

Taken together, this chapter demonstrates that deficiency of *recA* promotes the evolution of resistance to β-lactams antibiotics.

3.4 Reference

1. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science. 2004. 10;305(5690):1622-5.
2. Pu Y, Zhao Z, Li Y, Zou J, Ma Q, Zhao Y, Ke Y, Zhu Y, Chen H, Baker MAB, Ge H, Sun Y, Xie XS, Bai F. Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. Mol Cell. 2016 Apr 21;62(2):284-294.
3. Pu Y, Li Y, Jin X, Tian T, Ma Q, Zhao Z, Lin SY, Chen Z, Li B, Yao G, Leake MC, Lo CJ, Bai F. ATP-Dependent Dynamic Protein Aggregation Regulates Bacterial Dormancy Depth Critical for Antibiotic Tolerance. Mol Cell. 2019 Jan 3;73(1):143-156.e4.
4. Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., and McKinney, J.D. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. Science 339, 91–95.
5. Tobias Dörr, Kim Lewis, Marin Vulić. SOS Response Induces Persistence to Fluoroquinolones in Escherichia coli. PLoS Genet. 2009 Dec;5(12):e1000760.
6. Handwerger, S. & Tomasz, A. Antibiotic tolerance among clinical isolates of bacteria. Annu. Rev. Pharmacol. Toxicol. 25, 349–380 (1985).
7. McDermott, W. Microbial persistence. Yale J. Biol. Med. 30, 257–291 (1958).
8. Lederberg, J. & Zinder, N. Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc. 70, 4267–4268 (1948).
9. Cohen NR, Lobritz MA, Collins JJ. Microbial persistence and the road to drug resistance. Cell Host Microbe. 2013 Jun 12;13(6):632-42.
10. T. A. Leski and A. Tomasz: Role of penicillinbinding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J Bacteriol, 187, 1815–1824 (2005)
11. L. J. Sherrard, A. S. Tai, B. A. Wee, K. A. Ramsay, T. J. Kidd, N. L. Ben Zakour, D. M. Whiley, S. A. Beatson and S. C. Bell: Withinhost whole genome analysis of an antibiotic resistant Pseudomonas aeruginosa strain sub-type in cystic fibrosis. PLoS One, 12, e0172179 (2017)
12. R. Singh, K. R. Ledesma, K. T. Chang and V. H. Tam: Impact of recA on levofloxacin exposure-related resistance development. Antimicrob Agents Chemother, 54, 4262–4268 (2010)

13. J. W. Beaber, B. Hochhut and M. K. Waldor: SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature, 427, 72–74 (2004)
14. Alam MK, Alhhazmi A, DeCoteau JF, Luo Y2, Geyer CR. RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance. Cell Chem Biol. 2016 Mar 17;23(3):381-91.

Chapter 4. Role of SOS response in the evolution of resistance to β-lactams antibiotics

Chapter 4. Role of SOS response in the emergence of resistance to β-lactams antibiotics

4.1 Introduction

In the previous chapter (Chapter 3), I studied the role of *recA* in the emergence of resistance to β-lactams antibiotics. But more importantly, RecA has been demonstrated to be involved in the induction of SOS responses system. In a previous study, SOS system was reported to be required for the evolution from persistence to resistance induced by a single exposure of fluoroquinolones which damages the DNA structure. Therefore, in this chapter, the role of SOS response system will be explored. In particular, whether the induction of SOS response is required for the bacterial evolution of resistance induced by β-lactams antibiotics exposure.

4.1.1 Induction of SOS response in antibiotic resistance

The SOS response is a regulatory system which is widespread within bacterial with primary function is to detect and repair DNA damage induced by various environmental stress. DNA damage is resulted from the exposure to stressors such as antibiotics or UV exposure, increased oxidative stress, or nutrition restriction, causing single-stranded DNA (ssDNA) breaks [1]. Many features can be induced by SOS response, wherein one of the most important is the delay of cell cycle including the inhibition of cell division [2]. This inhibition provides bacterial cells sufficient time to repair DNA damage before committing to producing the two daughter cells, preventing potential deleterious effects.

The SOS response is regulated by two regulatory proteins which respectively control the SOS responses suppression and activation [3, 4]. LexA is a transcriptional regulator which represses over 40 genes in *Escherichia coli*. Thus, during normal cell growth, LexA is regarded as a SOS response regulon [5] which is achieved by LexA dimers binding to consensus DNA targets with a specific palindromic sequence known as LexA- or SOS-box [6]. When DNA undergoes damage, RecA will bind to the ssDNA breaks forming a RecA-ssDNA filament as well as known as an activated RecA (RecA*) to activate the induction of SOS response [7]. Subsequentially, the activated RecA (RecA*) stimulates the auto-proteolytic cleavage of LexA resulting in the derepression of LexA controlled genes and activation of the SOS regulon [8-10].

Not all LexA controlled genes are transcripted at the same time or extent in response to SOS induction or increased environmental stress. Many studies show that the LexA binding to SOS-boxes has different affinities depending on the number and location of the boxes relative to the promoters [11]. It is interesting that some of the genes can be induced to transcript early but some others are induced only when the DNA damage becomes more sever. In addition to this, the SOS response also occurs in a pulse-like pattern and as the response progresses, or more DNA damage occurs, the number of pulses increases [12], which implies that the SOS genes are regulated by multiple factors of induction to deal with DNA damage [13].

Genes regulated by LexA are mainly involved in the DNA repair, mutagenesis and arrest in cell division [1]. For example, gene *sulA* is responsible for the cell division inhibition in cell cycle, and the presence of SulA can result in the cell division delay and formation

of filaments, because SulA interplays with the essential cell division protein, FtsZ and prevents the polymerisation of FtsZ into a Z-ring [14-16].

4.1.2 Translesion synthesis polymerases are involved in SOS response

Bacteria produce translesion synthesis polymerases (TLS Pols) as part of the SOS response to DNA damage. They have historically been thought to serve as a last resort damage tolerance mechanism, re-starting replication forks that have stalled at damage sites on the DNA [17]. TLS Pols are highly error prone: inducing their activities leads to increased rates of mutation. It is now becoming clear that TLS Pols activity is an important source of mutations leading to de novo development of antibiotic resistance [28, 19]. For several species of bacteria, it has been shown that deleting genes for TLS Pols dramatically reduces rates of resistance development, and in some cases, even reduces infectivity [20-26]. Many of the drugs used to treat bacterial infections are known to increase mutation rates as a result of the drug triggering the SOS DNA-damage response and thus giving rise to an increase in the levels of TLS Pols. Most bacteria contain genes for TLS Pols, although the number and variety of these genes vary greatly. However, the key regulator of TLS Pols are highly conserved across the bacteria. Using currently available assays, in which resistant cells are identified by their ability to seed colonies on antibiotic-containing agar plates, it is impossible to discern if TLS Pols contribute to resistance by providing damage tolerance and increasing cell survival. Thereby, the chances that a resistant mutant will be found, or by facilitating adaptive mutation, selectively increasing mutation rates to speed the evolution of antibiotics resistance.

In both the damage tolerance and stress induced mutagenesis scenarios it is advantages for mutagenic pathways to be suppressed in low-stress environments and induced in high-stress environments. Taking the view that TLS Pols are maintained purely for their role in damage tolerance, mutagenesis is an unwanted by-product of rescuing stalled replication forks and thus TLS Pols activity should be restricted to times of dire need. Taking the adaptive mutation view, it has been theorized that while a high-mutation rate may be favourable under stress, it would be detrimental to cell fitness in low-stress conditions. Indeed, the activities of TLS Pols are tightly regulated inside cells. The best studied example of this regulation is in the *E. coli*, which contains five DNA Pols [68]. Two of these enzymes, Pol I and Pol III, are involved in normal DNA replication, copying the chromosome prior to cell division. The remaining three polymerases, Pol II, Pol IV and Pol V are TLS Pols and differ in their preference for various types of DNA lesions. All three are up-regulated as part of the SOS response [27]. Upon DNA damage, levels of Pol II increase from 30 to 350 molecules per cell [28], and from approximately 200 to 2000 for Pol IV [29]. Regulation of Pol V is even more stringent - it is rarely expressed in undamaged cells and reaches around 100 molecules per cell in UV-irradiated translational modifications, directed proteolysis, membrane-binding, and an internal ATPase activity that governs DNA binding [30].

4.1.3 Direct visualization of SOS response

Direct observation of the various activation steps of SOS response inside living cells with microscopy becomes important since it allows us to test various hypothesis *in vivo* bacterial cells. For example, the induction of SOS response can activate the enzyme DNA Pol V which is described as a TSL Pols. Whereas, DNA Pol V acting as a DNA repair

enzyme is produced through a series of steps dependent on nucleoprotein filaments of RecA*. Robinson *et al.* first demonstrate that UV exposure can induce the UmuC (DNA Pol V) production but not immediately activated. Instead, it was sequestered at the inner cell membrane. The release of UmuC into the cytosol requires the RecA*. The resulting delay in activation represents an additional molecular mechanism that limits the amount of time that this sometimes necessary but potentially detrimental enzyme spends on the DNA.

In addition to this, a recent report studied the spatial and temporal organization of RecA in *Escherichia coli* DNA-damage SOS response. Using a monomeric C-terminal fragment of the λ repressor as a novel fluorescent probe and single molecular microscopy they demonstrated that upon DNA damage, the storage structures dissolve and the cytosolic pool of RecA rapidly nucleates to form early SOS-signalling complexes, maturing into DNA-bound RecA bundles at later time points. Both before and after SOS induction, RecA* largely appears at locatins distal from replisomes, and upon completion of repair RecA storage structures can reform.

4.1.4 Bacterial cell division controlled by FtsZ

FtsZ is a highly conserved tubulin-like protein in bacteria that forms a ring-like structure (Z-ring) at the future division site (typically mid-cell). This ring acts as a scaffold for the assembly of the downstream divisome proteins and directs the synthesis of septal peptidoglycan [31, 32]. While the amino acid sequence of FtsZ shows little similarity to eukaryotic tubulin, their tertiary structures are very similar, suggesting they share common ancestry. Although the structure of *E. coli* FtsZ has not determined, structures

from several other species show FtsZ has a conserved fold [16-19]. This includes two independent domains, the N-terminal (NTD) and C-terminal (CTD) domains which are connected via a central helix [33]. The NTD contains the GTPase/ nucleotide binding site and the CTD contains the catalytic T7 loop as well as the disordered C-terminal tail, needed for the interaction of FtsZ with several division proteins [34, 35].

FtsZ monomers assemble in a linear head-to-tail manner to form short polymers known as protofilaments. Polymerisation is dependent on FtsZ monomers binding guanosine triphosphate (GTP). Upon binding GTP, the T7 loop of one FtsZ monomer inserts into the GTP binding domain of the next, activating the GTPase activity of FtsZ. The hydrolysis of GTP to GDP triggers the disassembly of the protofilaments, and drives the rapid turnover of polymeric and monomeric FtsZ [36].

FtsZ bound to the membrane by FtsA is able to deform the membrane in vitro in a GTP dependent manner [37, 38], suggesting that the Z ring provides at least part of the contractile force needed to pull the inner membrane during constriction [37, 39]. Recent advances in super resolution microscopy have revealed the Z ring to in fact be a discontinuous and heterogeneous distribution of FtsZ protofilaments throughout the ring [40, 41]. However the presence of the Z ring alone is not enough to provide constriction *in vivo* [42], indicating that later components of the divisome are also needed. It is now thought that Z ring dynamic treadmilling, and cell wall remodelling work together to facilitate constriction [43]. Treadmilling occurs when monomers preferentially bind to one end of a filament and dissociate from the other, causing the filament to move in the direction of preferential binding [43]. FtsZ protofilaments were first observed to treadmill on a lipid bilayer in vitro [44] and more recently this has been observed *in vivo* in both *B.*

subtilis and *E. coli* [45, 46]. These recent studies were a real breakthrough in this field, showing that treadmilling was dependent on GTPase activity and how this dynamic movement guided septal wall synthesis.

4.1.5 Antibiotics induced bacterial filamentation

Bacterial cells ranges from various shapes and sizes including rods, cocci, spirals and long cells. These variations in cells shape benefit bacterial cells with certain properties protecting themselves to survive against different environmental stress [47]. For example, being a small spherical cell in the presence of a predatory protozoan indicates that the cell may elude capture [48].

Antibiotics exposure such as β-lactams binding to penicillin binding proteins (PBP) or DNA damaging antibiotics fluoroquinolones can cause bacterial filamentation both *in vivo* and *in vitro* [49-51]. Whereas, these filamentous cells are able to resume division once the antibiotics are removed when they are exposed to sub-inhibitory concentration of antibiotics and induced to be filaments [52]. It is interesting that when re-exposed to a higher dosage of antibiotic treatment, these cells would be more resistant. These together imply that filamentation may be a transition state which drives bacterial evolution from persistence or tolerance to resistance to antibiotics.

Filamentation in *E. coli* induced by antibiotic is thought to be regulated through the activation of the increased stress and elevated mutation rates. For example, *E. coli* cells under sub-inhibitory concentrations of fluoroquinolones do not only form the filaments but also undergo asymmetrical division which can further be resistant to the same

concentrations of antibiotics. It is thereby believed that filamentation is the initial stage for bacterial cells to be resistance since it provides bacterial cells a sufficient time to generate and select for advantageous chromosomal mutations within the multinucleated filaments. However, I still do not fully understand the mechanisms giving rise to resistance during the formation of filaments, and even how bacterial resumes division when antibiotics are removed.

4.1.6 Chapter aims and objectives

The overall aim of this chapter was to understand the role of SOS response in the emergence of resistant in *ΔrecA* cells induced by a single exposure of β-lactam antibiotics. In particular, the first aim was to understand if the induction of SOS response is activated during the formation of resistance. This was achieved by our homemade super-resolution microscopy to direct observe in living cells.

The second aim was to understand if other SOS response associated genes are involved in the evolution of resistance, including *sulA, tolC,* and *ymfm*. This was achieved through a treatment of lethal concentration of ampicillin to various genetic modification strains, which can further quantify the co-maintenance of genes to infer functional proteins systems.

4.2 Results

4.2.1 Exposure of ampicillin triggers the formation of filaments

I first tested if exposure of ampicillin triggers the formation of filaments in wild type and *ΔrecA* cells. Cells were induced for gene modification and incubated with ampicillin at 50 μg/ml for 0, 4 and 8 hours, respectively. Microscopy was used to determine the cell size (for methods refer to Chapter 2).

Within this work, the term filamentation is defined to describe the cell growth and DNA replication in the absence or presence of antibiotics. In cells where the only effect is the complete inhibition of the cell division, the cell length is expected to double for each division generation per 30 minutes. This is measured by observing a shift in the mean cell length from one population to another (induced *v.s.* uninduced).

I found that after 0, 2, 4, 6 and 8 hours incubation with ampicillin, the length of bacterial cells in wild type and *ΔrecA* strains could be induced to increase from 1.78 ± 0.38 μm to 10.56 ± 4.85 μm and 1.65 ± 0.24 μm to 8.98 ± 3.65 μm indicating a formation of filaments (Fig. 4.1).

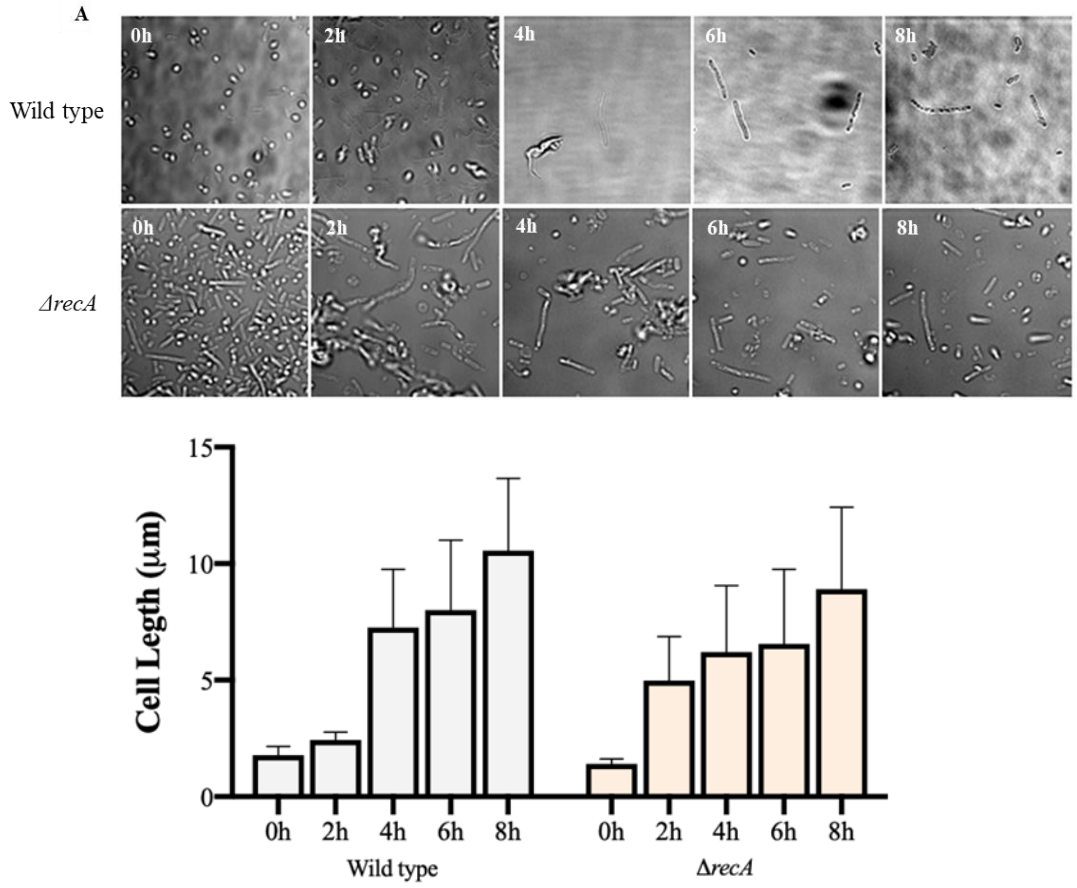


Figure 4.1 Morphological changes of E. coli cells exposed to ampicillin at lethal concentration. *Wild type and ΔrecA strains in LB medium were exposed to 50 μg/ml ampicillin and incubated for 0, 2, 4, 6 and 8 hours. After the incubation, cells were fixed and applied on a gel pad to be imaged using TIRF microscopy at 100 × magnification under phase contrast. Cell length was determined by the method refer to Chapter 2. Scale bar = 2 μm.*

4.2.2 Emergence of Multinucleated bacterial filaments

Multinucleated bacterial filaments were observed previously when bacterial cells were exposed to low level of ciprofloxacin (0.125 × MIC) (data not published). Here, I found

that after 4 and 8 hours treatment by ampicillin at lethal concentration (10 × MIC), bacterial cells both in wild type and *ΔrecA* strains could be induced to be multinucleated filaments as well. In particular, some chromosomes localized near the tips, coinciding with the position of the division sites at the tips. STORM imaging showed that without the treatment of ampicillin either in wild type or *ΔrecA* strain two individual chromatins were eventually observed to alternate between overlapping in a single cluster and then separating into two distinct foci, but in filaments multiple chromatins undergo replication or separation of foci were formed as an asynchronous way (Fig. 4.2).

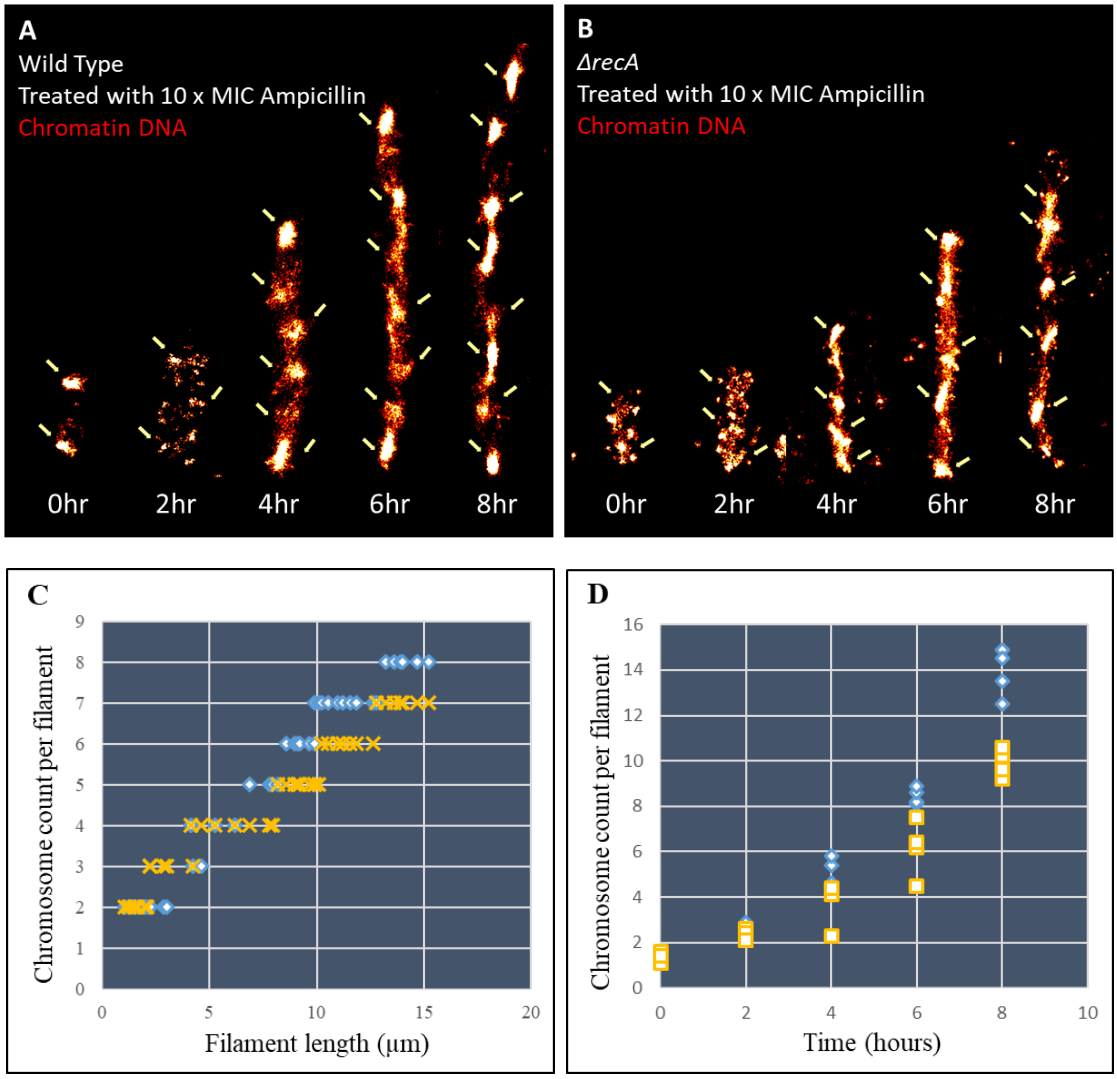


Figure. 4.2 Emergence of multinucleated bacterial filaments. *Wild type and ΔrecA strains were incubated with lethal concentration of ampicillin (10 ×MIC) for 0, 2, 4, 6, and 8 hours respectively. After the killing, cells were fixed to be imaged. (A and B) STORM images of chromosome showing dynamic changes of individual chromosomes along the filament length (Scar bar = 2 µm). Each red circle loci denotes a chromosome and results from automated analysis software (methods refer to Chapter 2). Yellow arrowhead indicates a chromosome separation event. (C) Chromosome count as a function of bacterial cell length as measure by EdU staining. (D) Distribution of chromosome loci position in individual cells shown in filaments measured by an*

automated analysis software (methods refer to Chapter 2). Blue dots represent wild type strains. Yellow dots represent ΔrecA strains.

4.2.3 Reduced induction of SOS response during the emergence of resistance

The induction of SOS response was believed to be involved in the emergence of resistance. I thereby tested whether the single exposure of β-lactam antibiotics induces the responses of SOS system. STORM images with spatial resolution of 25nm revealed that after 0, 2, 4, 6, and 8 hours ampicillin treatment co-localization ratio between the chromatin and DNA polymerase I was dramatically increased in wild type *E. coli* strain showing that the induction of SOS responses since the DNA polymerase I was induced to move from cell membrane to cytoplasm binding to DNA for DNA repair (Fig. 4.3A and C). However, in *ΔrecA* strain, this ratio was significantly reduced from 80% to 57%, indicating that the induction of SOS response was inhibited in cells without *recA* expression (Fig. 4.3B and C).

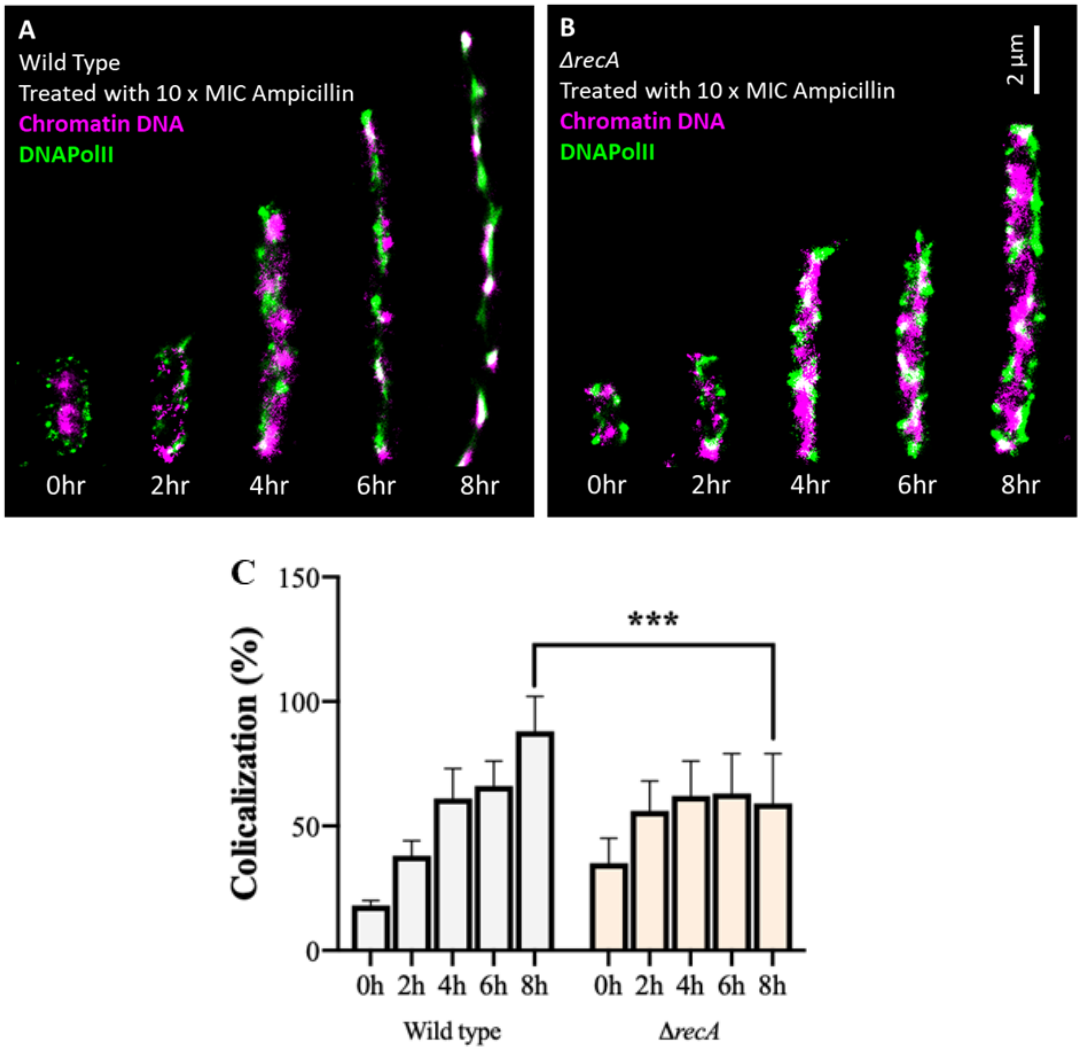


Figure 4.3 Induction of SOS response is reduced in ΔrecA strain. *(A and B) Dual color STORM images reveal a movement of DNA polymerase I from cell membrane to cytoplasm binding to DNA. In ΔrecA strains, this movement was inhibited as the colocalization of two signals was reduced indicating a reduced induction of SOS response. Scan bar = 2 μm. (C) Statically calculation of the co-localization between bacterial chromosome and DNA polymerase I. Each group contains bacterial cell number > 200. *p < 0.05; **p < 0.01; ***p < 0.001. Mann-Whitney rank sum test.*

4.2.4 Induction of SOS response is not required for β-lactams antibiotics induced formation of resistance

For I found that ampicillin induced SOS response induction was reduced in *ΔrecA* strain but its exposure was able to trigger the evolution of resistance, I asked whether the SOS induction was required for the antibiotic resistant evolution. To test the role of SOS response, I used three more genetic modification strains: first, *ΔlexA* of which SOS response is always switched on; second, $lexA^{ind-}$ strains whose SOS response is always switched off; third, *ΔsulA* strain in which the induction of SOS response is inhibited.

After a single exposure of ampicillin at 50 μg/ml, I found that none of these strains exhibited resistance to ampicillin indicating that neither the induction nor inhibition of SOS response was not involved in the evolution of antibiotic resistance induced by β-lactams antibiotics exposure (Fig. 4.4). It is interesting that RecA is usually believed to participate in the induction of SOS response. However, I here found that β-lactams antibiotic exposure induced antibiotics resistant evolution was not dependent on the SOS response induction, which will be discussed more in next chapter.

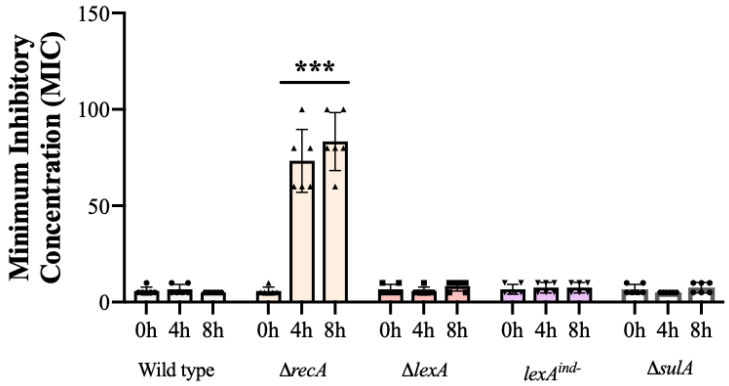


Figure 4.4 Evolution of resistance induced by β-lactams antibiotics exposure is not dependent on the SOS induction. *MICs of E. coli wild type, ΔrecA, ΔlexA, lexAind- and ΔsulA strains batch cultures after the ampicillin treatment for 0, 2, 4, 6 and 8 hours, respectively. All data are presented as the mean ± s.d. of six independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. Mann-Whitney rank sum test.*

4.2.5 FtsZ assembly was disrupted in bacterial filaments

The disruption of FtsZ assembly is demonstrated to induce the bacterial morphology change, which can cause cell division arrest and formation of filaments. I first used time-lapse microscopy to monitor the bacterial cell division and FtsZ assembly in wild type cells in the absence of antibiotics. I found that bacterial cells without the treatment of antibiotics were divided every 60 minutes under the certain experimental condition, and FtsZ could be assembled in the middle of each cell in prior to the division (Fig. 4.5A).

However, in the presence of antibiotics (ampicillin at 50 μg/ml), bacterial cells were induced to be filaments, but the FtsZ assembly was significantly disrupted, because I observed that the signal of FtsZ was randomly distributed in cells (Fig. 4.5B). I also

observed some dormant cells survived under the treatment of ampicillin, and more interestingly in dormant cells the signal of FtsZ could not be detected which may imply that the expression of FtsZ was inhibited as a unknown survive strategy protecting bacterial cells against the antibiotic lethality (Fig. 4.5C).

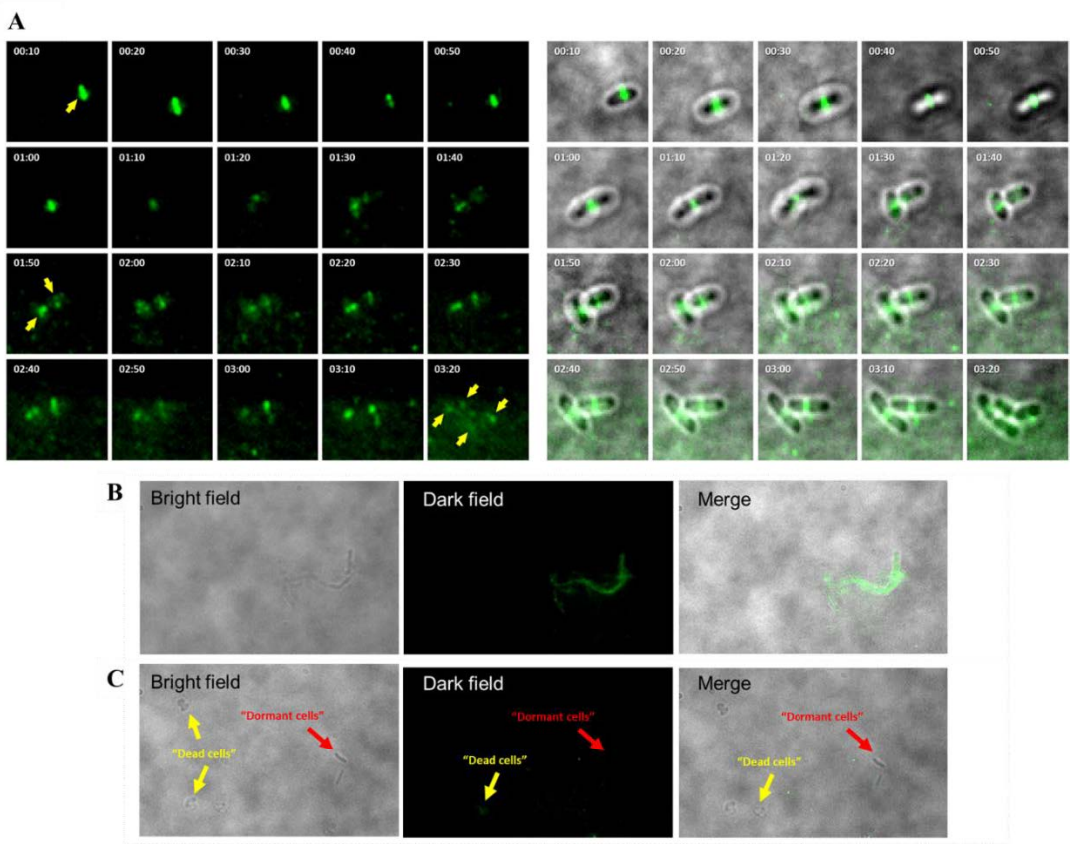


Figure 4.5 FtsZ assembly was disrupted in filaments. *(A) Time-lapse microscopy shows the assembly of FtsZ during the bacterial cell division in the absence of ampicillin. (B) Assembly of FtsZ was disrupted in bacterial filmanets. (C) The expression of FtsZ was inhibited in survived dormant cells in the presence of antibiotics.*

4.2.6 Exposure of ampicillin induces formation of uncharacterized round cells in SOS response arrested cells

In addition to the filamentation, I observed round morphology in bacterial cells after β-lactams treatment. Fig. 4.6A shows that without the treatment of ampicillin, *E. coli* cells are rod shaped; however, after 8 hours exposure, in all populations including wily type, *ΔrecA, ΔsulA, Δymfm,* and *ΔtolC*, bacterial cells can be induced to be round-shaped morphology.

It is interesting that round-shaped cells would be resume to be rod-like morphology after an overnight culture in fresh medium (Fig. 4.6B). Although I have no idea about what regulates the morphology changes during the evolution of resistance in wild type or SOS response arrested cells, previous findings reported that overexpression of a gene *bolA* promotes the morphology changes from rod-shaped to round-shaped both in M9 minimal medium and Luria broth rich medium. Currently I have no evidence supporting the morphology changes observed in our experiments resulted from the overexpression of *bolA*, whereas I am planning to study the function of *bolA* in the emergence of resistance in future.

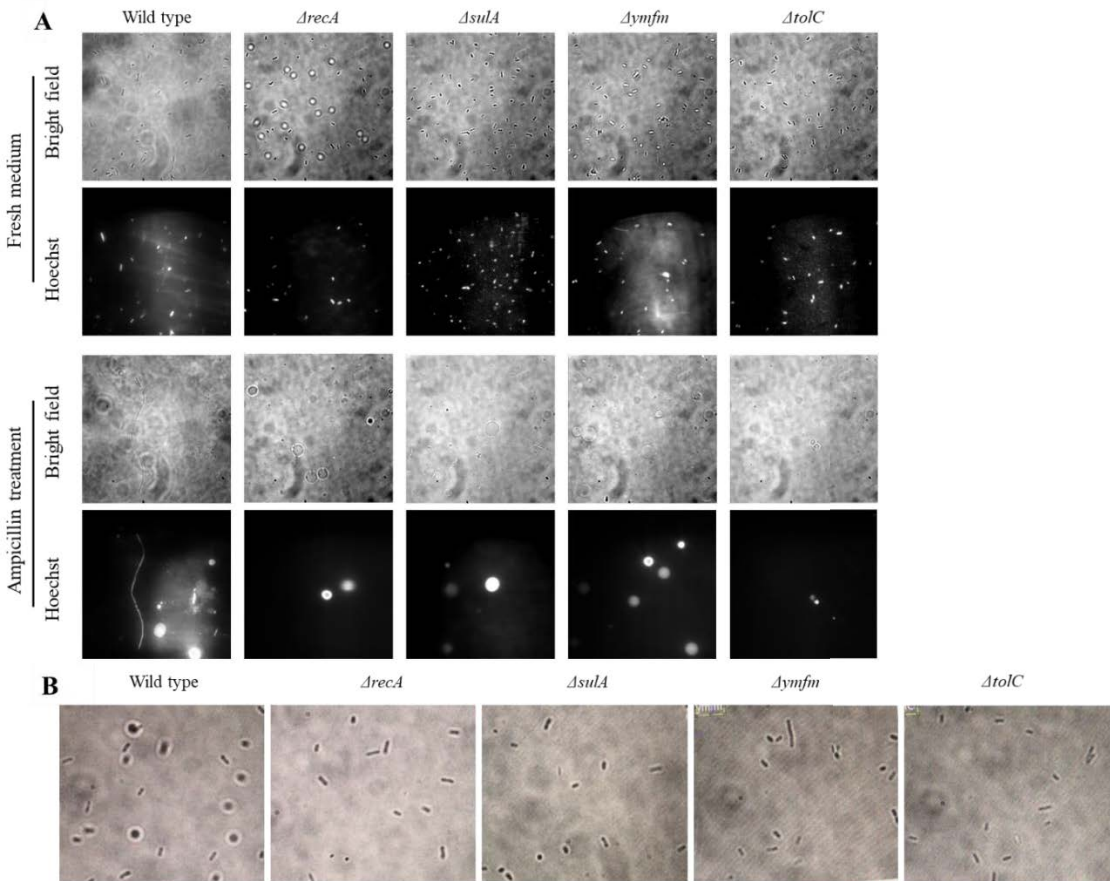


Figure 4.5 Morphology changes in SOS response arrested cells. *(A) All bacterial cells were incubated with ampicillin at 50 μg/ml for 8 hours and then fixed to be imaged. TIRF microscopy reveals that during the killing, rod-like cells could be induced to change to round-shaped cells in SOS arrested strains, including ΔrecA, ΔsulA, Δymfm, and ΔtolC strain. (B) Round-shaped cells could resume to be rod-like cells after an overnight culture in fresh medium. Scar bar = 1μm.*

4.3 Conclusions

The research in this chapter was performed to initiate the investigation into what the role of SOS response in the bacterial evolution of resistance to antibiotics induced by the exposure of β-lactams antibiotics.

Using super resolution microscopy – STORM I found that in *ΔrecA* strain the induction of SOS response was reduced demonstrating that *recA* was required for the activation of SOS response. However, on contrast to previous findings, exposure of β-lactams antibiotics induced emergence of resistance which was not dependent on the induction of SOS response, and in *ΔrecA* resistant isolates I observed that the SOS induction was significantly inhibited as well.

On the other hand, multinucleated filamentation was observed indicating that cells division was inhibited. Cell filamentation during SOS is thought to reflect the need to complete DNA replication before cell division to protect chromosomes after DNA damage. Similarly, filamentation allows repair of DNA damage by recombination between chromosomes by keeping more than one chromosome per cell until repair is completed**.** I suggest that in addition to DNA preservation, cell filamentation may accelerate evolution by adding recombination, which is possible only with more than one chromosome per cell, to the increased mutagenesis documented.

Although I found these observations captivating, it remains unclear to us why there is such heterogeneity in the chromosome dynamics. One can speculate that the nonstationary chromosomes are the chromosomes with increased fitness (probably containing mutations conferring cipro resistance) that would not be eliminated but chosen for replication. Instead, stationary and/or nonreplicating chromosomes that are likely to contain neutral or deleterious mutations would eventually be eliminated in the dying filament or would recombine to separate beneficial mutations from linked deleterious damage and hence be chosen for survival.

These results taken together demonstrate that the induction of SOS response is not involved in the single exposure of β-lactams antibiotics triggered evolution of antibiotic resistance.

4.4 Reference

1. Simmons, L.A., et al., The SOS Regulatory Network. EcoSal Plus, 2008. 3(1).
2. Huisman, O., R. D'Ari, and S. Gottesman, Cell-division control in Escherichia coli: specific induction of the SOS function SfiA protein is sufficient to block septation. Proc Natl Acad Sci U S A, 1984. 81(14): p. 4490-4.
3. Little, J.W., Mechanism of specific LexA cleavage: autodigestion and the role of RecA coprotease. Biochimie, 1991. 73(4): p. 411-21.
4. Little, J.W., The SOS regulatory system: control of its state by the level of RecA protease. J Mol Biol, 1983. 167(4): p. 791-808.
5. Butala, M., D. Zgur-Bertok, and S.J. Busby, The bacterial LexA transcriptional repressor. Cell Mol Life Sci, 2009. 66(1): p. 82-93.
6. Chen, Y., S.L. Milam, and H.P. Erickson, SulA Inhibits Assembly of FtsZ by a Simple Sequestration Mechanism. Biochemistry, 2012. 51(14): p. 3100-9.
7. Chen, Z., H. Yang, and N.P. Pavletich, Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. Nature, 2008. 453(7194): p. 489-4.
8. Giese, K.C., C.B. Michalowski, and J.W. Little, RecA-dependent cleavage of LexA dimers. J Mol Biol, 2008. 377(1): p. 148-61.
9. Kovacic, L., et al., Structural insight into LexA-RecA* interaction. Nucleic Acids Res, 2013. 41(21): p. 9901-10.

10. Gruenig, M.C., et al., RecA-mediated SOS induction requires an extended filament conformation but no ATP hydrolysis. Mol Microbiol, 2008. 69(5): p. 1165-79.
11. Courcelle, J., et al., Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient Escherichia coli. Genetics, 2001. 158(1): p. 41-64.
12. Friedman, N., et al., Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. PLoS Biol, 2005. 3(7): p. e238.
13. Michel, B., After 30 years of study, the bacterial SOS response still surprises us. PLoS Biol, 2005. 3(7): p. e255.
14. Janion, C., Inducible SOS response system of DNA repair and mutagenesis in Escherichia coli. Int J Biol Sci, 2008. 4(6): p. 338-44.
15. Cordell, S.C., E.J. Robinson, and J. Lowe, Crystal structure of the SOS cell division inhibitor SulA and in complex with FtsZ. Proc Natl Acad Sci U S A, 2003. 100(13): p. 7889-94.
16. Dajkovic, A., A. Mukherjee, and J. Lutkenhaus, Investigation of regulation of FtsZ assembly by SulA and development of a model for FtsZ polymerization. J Bacteriol, 2008. 190(7): p. 2513-26.
17. Goodman, M. F. & R. Woodgate, Translesion DNA polymerases. Cold Spring Harb Perspect Bio. 10, p. a010363 (2013).
18. Andersson, D. I., S. Koskiniemi, & S. E. Finkel. Biological roles of translesion synthesis DNA polymerases in eubacteria. Mol Microbiol. 77, 540-548 (2010)
19. Corzett, C.H., M.F. Goodman, & S.E. Finkel, Competitive fitness during feast and famine: how SOS DNA polymerases influence physiology and evolution in Escherichia coli. Genetics. t94(2): 409-20 (2013).

20. Aranda, J., et al., Role of Acinetobacter baumannii UmuD homologs in antibiotic resistance acquired through DNA damage-induced mutagenesis. Antimicrob Agents Chemother. 58(3): 77l-773 (2014).
21. Boshoft H.I., et al., DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. Cell.113(2): 183-193 (2013).
22. Cirz, R.T. & F.E. Romesberg. Controlling mutation: intervening in evolution as a therapeutic strategy. Crit Rev Biochem Mol Biol, a2: p.341-354 (2017).
23. Henderson-Begg, S.K., D.M. Livermore, Z & L.M. Hall, Effect of subinhibitory concentrations of antibiotics on mutaĺion frequency in Streptococcus pneumoniae. J Antimicrob Chemother,7(5): 849-854 (2016).
24. Sanders, L.H., et al., Role of Pseudomonas aeruginosa dinB-encoded DNA polymerase IV in mutagenesis. J Bacteriol.188(24): 8573-85 (2006).
25. Torres-Barcelo, C., et al., The SOS response increases bacterial fitness, but not evaluability, under a sublet hat dose of antibiotic. Proc Biol Sci.282(1816): p. 20150885 (2015).
26. Warner, D.F., et al., Essential roles for imuA- and imuB-encoded accessory factors in DnaE2- dependent mutagenesis in Mycobacterium tuberculosis. Proc Natl Acad Sci USA. 107(29): I3093-3098 (2010).
27. Goodman, M.F., Error-prone repair DNA polymerases in prokaryotes and eukaryotes. Annu Rev Biochem. 7: l7-50 (2002).
28. Qiu, Z. and M.F. Goodman, The Escherichia coli polB locus is identical to dinA, the structural gene for DNA polymerase II. Characterization of Pol II purified from a polB mutant. J Biol Chem, 272(13): 86ll-8617 (1997).

29. Kim, S.R., et al., Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutagenesis in Escherichia coli. Mol Genet Genomics.266(2): 207-215 (2001).

30. Robinson A., et a1., Regulation of Mutagenic DNA Polymerase V Activation in Space and Time. PLoS Genet. 11(8): p. e1005482 (2015).

31. Bi, E.F. and J. Lutkenhaus, FtsZ ring structure associated with division in Escherichia coli. Nature, 1991. 354(6349): p. 161-4.

32. Schoenemann, K.M. and W. Margolin, Bacterial Division: FtsZ Treadmills to Build a Beautiful Wall. Curr Biol, 2017. 27(8): p. R301-R303.

33. Lowe, J. and L.A. Amos, Crystal structure of the bacterial cell-division protein FtsZ. Nature, 1998. 391(6663): p. 203.

34. Ma, X. and W. Margolin, Genetic and functional analyses of the conserved C-terminal core domain of Escherichia coli FtsZ. J Bacteriol, 1999. 181(24): p. 7531-44.

35. Mosyak, L., et al., The bacterial cell-division protein ZipA and its interaction with an FtsZ fragment revealed by X-ray crystallography. EMBO J, 2000. 19(13): p. 3179-91.

36. Anderson, D.E., F.J. Gueiros-Filho, and H.P. Erickson, Assembly dynamics of FtsZ rings in Bacillus subtilis and Escherichia coli and effects of FtsZ-regulating proteins. J Bacteriol, 2004. 186(17): p. 5775-81.

37. Osawa, M. and H.P. Erickson, Inside-out Z rings--constriction with and without GTP hydrolysis. Mol Microbiol, 2011. 81(2): p. 571-9.

38. Osawa, M. and H.P. Erickson, Liposome division by a simple bacterial division machinery. Proc Natl Acad Sci U S A, 2013. 110(27): p. 11000-4.

39. Erickson, H.P., D.E. Anderson, and M. Osawa, FtsZ in bacterial cytokinesis: cytoskeleton and force generator all in one. Microbiol Mol Biol Rev, 2010. 74(4): p. 504-28.

40. Strauss, M.P., et al., 3D-SIM super resolution microscopy reveals a bead-like arrangement for FtsZ and the division machinery: implications for triggering cytokinesis. PLoS Biol, 2012. 10(9): p. e1001389.

41. Rowlett, V.W. and W. Margolin, 3D-SIM super-resolution of FtsZ and its membrane tethers in Escherichia coli cells. Biophys J, 2014. 107(8): p. L17-20.

42. Daley, D.O., U. Skoglund, and B. Soderstrom, FtsZ does not initiate membrane constriction at the onset of division. Sci Rep, 2016. 6: p. 33138.

43. Schoenemann, K.M. and W. Margolin, Bacterial Division: FtsZ Treadmills to Build a Beautiful Wall. Curr Biol, 2017. 27(8): p. R301-R303.

44. Loose, M. and T.J. Mitchison, The bacterial cell division proteins FtsA and FtsZ self-organize into dynamic cytoskeletal patterns. Nat Cell Biol, 2014. 16(1): p. 38-46.

45. Bisson-Filho, A.W., et al., Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science, 2017. 355(6326): p. 739-743.

46. Yang, X., et al., GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. Science, 2017. 355(6326): p. 744-747.

47. Yang, D.C., K.M. Blair, and N.R. Salama, Staying in Shape: the Impact of Cell Shape on Bacterial Survival in Diverse Environments. Microbiol Mol Biol Rev, 2016. 80(1): p. 187-203.

48. Young, K.D., The selective value of bacterial shape. Microbiol Mol Biol Rev, 2006. 70(3): p. 660-703.

49. Comber, K.R., R.J. Boon, and R. Sutherland, Comparative effects of amoxycillin and ampicillin on the morphology of Escherichia coli in vivo and correlation with activity. Antimicrob Agents Chemother, 1977. 12(6): p. 736-44.
50. Elliott, T.S., A. Shelton, and D. Greenwood, The response of Escherichia coli to ciprofloxacin and norfloxacin. J Med Microbiol, 1987. 23(1): p. 83-8.
51. Suzuki, H., J. Pangborn, and W.W. Kilgore, Filamentous cells of Escherichia coli formed in the presence of mitomycin. J Bacteriol, 1967. 93(2): p. 683-8.
52. Chen, K., et al., Modified virulence of antibiotic-induced Burkholderia pseudomallei filaments. Antimicrob Agents Chemother, 2005. 49(3): p. 1002-9.

Chapter 5. *pinR* determines the bacterial evolution of resistance to β-lactams antibiotics

Chapter 5. *pinR* determines the bacterial evolution of resistance to β-lactams antibiotics

5.1 Introduction

5.1.1 Gene mutation in the emergence of resistance

Bacterial antibiotic resistance can be achieved by genetic regulation, including horizontal acquisition of resistance genes, recombination of foreign DNA into chromosome, and mutations in different chromosomes [1]. In the presence of antibiotics, bacterial cells tend to acquire gene mutations through an adaptive mutation pathway in which the mutation process usually occurs in dividing bacterial cells, because it is regarded as a consequence of errors during the DNA replication process [2, 3]. For example, it has been demonstrated that quinolone antibiotics can induce the SOS mutagenic response and consequently increase the mutation rate and emergence of resistance to these antibiotics [4]. DNA mutations occurring on MDR is reported to increase the antibiotic resistance in *P. aeruginosa* [5]. Under the exposure of streptomycin, *E. coli* can display a hypermutable phenotype [6]. Moreover, starvation conditions can increase the mutation rate of *Salmonella enterica* to rifampin resistance. All of these results demonstrate that adaptive gene mutation has a significant effect on the bacterial growth and survive.

Independent mutation is another important mechanism for bacterial acquiring gene mutations and resulting in antibiotic resistance including target structural mutations and subunits mutations. For example, exposure of quinolones can cause the mutations on the genes that encode protein GyrA or GyrB subunit of topoisomerase II leading to the

emergence of phenotypes of resistance [7]. In some other cases, bacterial cells can acquire resistance through increasing the gene mutation rate on the genes which are required for the access of antibiotics to their targets, for instance, the treatment under sub-lethal concentration of quinolone can induce the emergence of resistance in *Pseudomonas aeruginosa* which is achieved by gene mutations occurring on the MDR determinants [8].

5.1.2 Regulation of *ampC* in *E. coli*

AmpC β-lactamases are active-site serine enzymes that most significantly induce the bacterial evolution of resistance under the exposure of β-lactams antibiotics. In many Gram-negative organisms including *E. coli* the expression of chromosomal *ampC* gene is generally low but can be induced in response to the treatment of β-lactams antibiotics or other stimuli [9]. Induction of *ampC* is regulated by several gene products associated with the cell wall synthesis pathway including gene *ampR, ampD, ampG,* and *ampE* (Fig. 5.1).

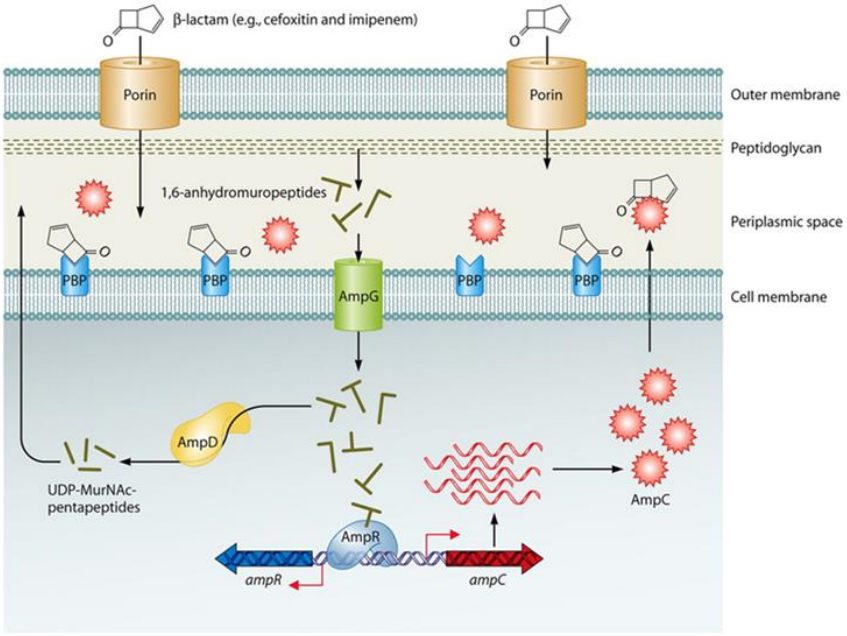


Figure 5.1 Mechanism involved in regulation of ampC expression. *β-Lactams antibiotics induction of ampC expression. Inducing β-lactams, such as cefoxitin and imipenem, cross the outer membrane through porins, enter the periplasmic space, and interact with target peinicillin binding proteins (PBPs). An increase in pools of 1,6-anhydromuropeptides is observed, and AmpD is unable to efficiently process the higher levels of cell wall fragments. The anhydro-MurNAc-peptides (inducing peptides) replace UDP-MurNAc-pentapeptides (suppressing peptides) bound to AmpR, causing a conformational change in the protein. AmpR is converted into a transcriptional activator, ampC is expressed at higher levels, and levels of AmpC increase in the periplasmic space. When the amount of β-lactam decreases below "inducing levels," the cytoplasmic pool of anhydro-MurNAc-peptides also decreases, and AmpD is able to efficiently cleave these peptides, restoring wild-type ampC expression [9].*

ampR. AmpR is a transcriptional regulator of *ampC* and its gene *ampR* is located between *ampC* and the fumarate reductase operon which promotes the *ampR* and *ampC* RNA

transcription (Fig. 5.1). The induction of *ampC* is depended on the cofactor which is shown to bind to *ampR* at the time of DNA binding. Specific mutations occurring on *ampR* is reported to cause noninducible, high constitutive, and hyperinducible *ampC* expression, which are associated with the efficiency of DNA binding, because DNA binding is important for protein-protein interactions required for the transcriptional activation.

ampD. AmpD is demonstrated to be responsible for the overexpression of *ampC* outside of the region encoding the *ampC* and *ampR* genes (Fig. 5.1). Meanwhile, the constitutive or semi-constitutive induction of overexpression of *ampC* is conferred by the mutations occurring on the *ampD* but which is found to be *ampR*-dependent. Genetic analysis confirms that *ampD* mutations result in a different *ampC* induction, for instance semi-constitutive, stably derepressed, hyperinductible and temperature-sensitive *ampC* expression. The role of *ampD* is associated with the recycling of cell wall components during the cell wall synthesis, thus *ampD* mutations can cause the change of kinetics of cell wall metabolites and consequently protect bacterial cells against antibiotics lethality.

ampG. AmpG is a transporter of muropeptide from the periplasmic space to the cytosol (Fig. 5.1), but in contrast to *ampD*, *ampG* is shown to have no influence on *ampC* induction. However, studies demonstrate that *ampG* is playing a role in the cell wall recycling pathway which is responsible for the transportation of oligopeptides that is required for the β-lactamase transcription induced by *ampC*.

5.1.3 Gene duplication and amplification in bacterial evolution of antibiotic resistance

Gene duplication and amplification are genetic pathways through which new genetic material is generated and inserted in whole chromosome. In addition to gene specific mutations, gene duplication and amplification have also been shown to constitute an important adaptive mechanism in bacterial evolution of resistance induced by antibiotic exposure. For example, resistance to sulphonamide and trimethoprim can be conferred by increased gene dosage through gene duplication and amplification of antibiotic hydrolytic enzymes, target enzymes or efflux pumps. Furthermore, most types of antibiotic resistance mechanism are deleterious in the absence of antibiotics, and these fitness costs can be ameliorated by increased gene dosage of limiting functions.

Normark *et al*. first reported that chromosomal gene amplification can cause an increased β-lactams antibiotics resistance. They found that *E. coli* strain with artificial genetic modification containing multiple copies numbers of *ampC* (~30 copies) could increase the resistance to ampicillin. More importantly, this increased resistance was isolated in both *recA*$^{-}$ or *recA*$^{+}$ strains; however, *recA*$^{-}$ mutations could only stabilize the amplification and high-level resistance. Further analysis demonstrated that the duplication join points did not originate from any large regions of sequence homology but from different short (12–13 bp) direct repeats, and the initial duplications probably occurred through recA-independent mechanisms (10, 11).

5.1.4 Chapter aims and objectives

The overall aim of this chapter was to understand whether the single exposure of ampicillin induced resistance was resulted from genes mutations. In particular, the first aim was to investigate if the gene *ampC* and its regulated genes including *ampG, ampD, ampR,* and *ampE* were mutated, because I found there coding protein β-lactamase expression was up-regulated in resistant isolates. This was achieved by exploring the whole genome sequencing.

The second aim was to find if there was other gene mutations contributing in the emergence of resistance. This was achieved by whole genome sequencing and droplet digital PCR measurement.

The third aim was to figure out if bacterial gene duplication and amplification participated in the induction of bacterial evolution of resistance. This was achieved by the droplet digital PCR measurement.

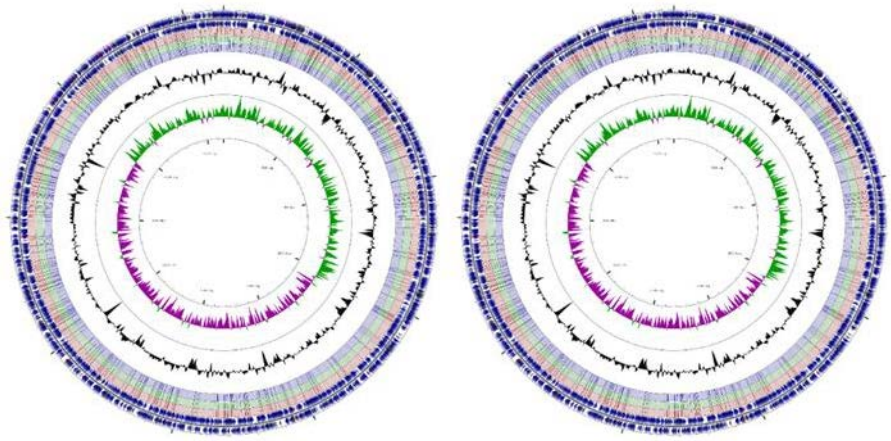
5.2 Results

5.2.1 Exposure of ampicillin triggers *pinR* mutations

Many studies demonstrated that SNPs mutation on gene *ampC* or its promoter could largely increase the β-lactamase expression providing highly resistance to β-lactam antibiotics. I examined a whole genome sequencing to figure out if there were gene mutations occurring on *ampC* or its promoter. It is interesting that I did not found any

SNPs mutation that were occurred on *ampC* and even its regulation associated genes, including *ampR, ampG, ampD,* and *ampE* (Fig. 5.2) which indicates that the higher expression of β-lactamase was not mediated by the *ampC* gene mutations.

I further analysed other gene sequencing and found that three SNPs occurred in an uncharacterized gene named *pin*. In particular, first, a G > A mutation in base 1,433,667 of the *E. coli* genome caused an Arg > Gln mutation; Second, a T > C mutation in base 1,433,199 of the *E. coli* genome caused a Val > Ala mutation. Third, a T > A mutation in base 1,433,228 of the *E. coli* genome caused a synonymous mutation (Fig. 5.1).



Wild type *ΔrecA*

Gene mutations

Strains	Feature	Position	Mutation
Wild type			
Wild type 4h	*pinR*	1433228	T > A
		1433199	T > C
Wild type 8h	*pinR*	1433228	T > A
		1433199	T > C
		1433667	G > A
ΔrecA			
ΔrecA 4h	*pinR*	1433667	G > A
ΔrecA 8h	*pinR*	1433228	T > A
		1433199	T > C
		1433667	G > A

Figure 5.1. Summary of E. coli whole genome sequencing. *Six genomes were sequenced: the wild type strain without treatment, two tolerant isolates evolved from wild type strain, ΔrecA strain without treatment, two resistant isolates evolved from ΔrecA strain. Sequencing coverage for each isolate is plotted. Three SNPs were found in four strains occurring on the gene pinR.*

5.2.2 Protein structure and function prediction of PinR

PinR is a serine recombinase with no determined structure and function. Interpro analysis reveals that PinR belongs to 'resolvase' family which share the following structural characteristics: an N-terminal catalytic and dimerization domain that contains a conserved serine residue involved in the transient covalent attachment to DNA and a C-terminal helix-turn-helix DNA-binding domain (Fig. 5.2A).I further used an online Phyre^2 modelling serve by homology modelling under “intensive” mode (Kelley et al., 2015) to predict the protein 3D modelling and secondary structure of PinR. The following three PDB templates including c2gm4B (PDB Title: an activated, tetrameric gamma-delta resolvase: hin chimaera bound to2 cleaved dna), c4m6fA (PDB Title: dimer of the g-segment invertase bound to a dna substrate dna-invertase), and c2r0qF (PDB Title: crystal structure of a serine recombinase -DNA regulatory 2 complex) were selected to model the protein based on heuristics to maximise confidence, percentage identity and alignment coverage. These templates had ~ 30% identity respectively with the final model that at least 97% residues were modelled with at least 90% confidence (Fig. 5.2B).

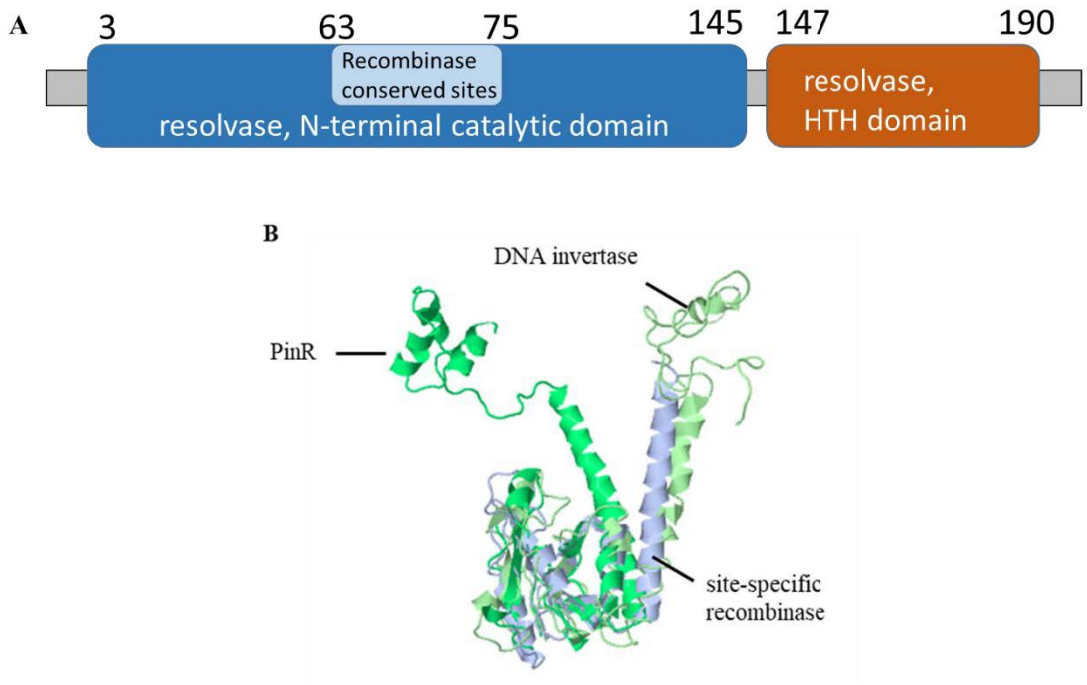


Figure 5.2 PinR structure prediction. *(A) Interpro analysis reveals that PinR belongs to 'resolvase' family with an N-terminal catalytic domain and C-terminal helix-turn-helix DNA-binding domain. (B) Comparison of the protein structure among predicted protein PinR (phyre[2]), protein DNA invertase, and protein site-specific recombinase structure.*

The ribbon diagrams of the three-dimensional structure were prepared using Swiss-Pdb Viewer v4.1.0. The α-helices are shown in red and the β-sheets in blue (Fig. 5.3). The mutations R3Q (yellow spheres) and V160A (green spheres) appear to not have significant effect on the protein overall tertiary structure, but V160A is predicted to alter the local secondary structure by decreasing the one sheet region and two alpha-helix regions (Fig. 5.3A). Structural predictions of residual location and interactions reveal that ALA160 in pinR_V160A is located at an alpha-helix region, and forms stronger hydrogen bonds with LYS157 (distance 2.44 Å) and ARG163 (distance 2.52 Å), which may increase hydrogen bond network and therefore stabilizing the local region (Fig. 5.3B and

C). Substitutions may contributes to some extent to its overall stability and/or activity and may form a delicate network of multiple interactions with its neighbours.

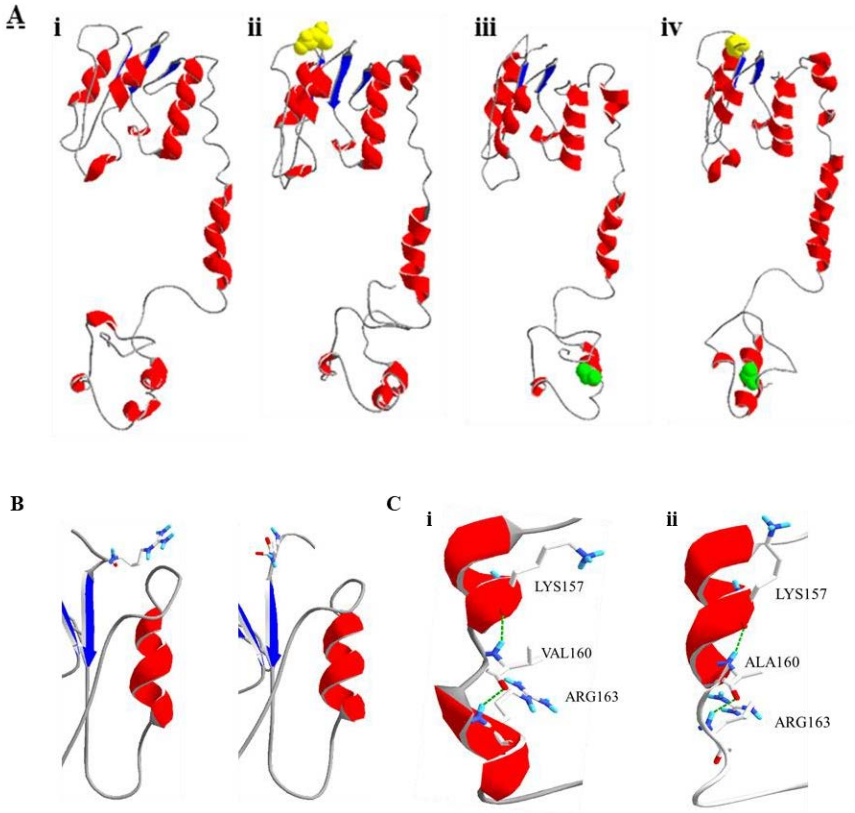


Figure 5.3 Secondary structure prediction of PinR and its mutants. *(A) Homology-based modeling of PinR. The ribbon diagrams of the three-dimensional structure were prepared using Swiss-Pdb Viewer v4.1.0. The α-helices are shown in red and the β-sheets in blue. i, PinR_wild type; ii, PinR_R3Q; iii, PinR_V160A; iv, PinR R3Q/V160A. (B) Structural predictions of residual location and interactions in wild-type PinR and mutated PinR predicted by Swiss-PDB viewer v4.1.0. i, PinR_wild type; ii, PinR_R3Q. (C) Structure of hydrogen bonds predictions. i, PinR_wild type; ii, PinR_V160A.*

The optimal model of each protein was submitted to the 3DLigandSite server to predict potential active sties (Wass et al., 2010). Supplemental Table 5.1 lists predicted residues responsible for forming active sites and heterogens, and Figure 5.4 depicts the predicted active site with binding heterogens which implies that mutations R3Q and V160A may increase the numbers of binding sites and consequently elevate the binding efficiency of protein PinR.

Table 5.1 3DLigandsite active site predictions

Protein	*Predicted Binding site*	*Heterogens*
pinR_WT	AGR9, ILE10, SER11, THR12, GLN15, THR16, ASN19, GLN20, LYS70, ARG73	COA, GAL, ADP, FMN, MG, CA
PinR_R3Q	SER11, ASP72, ARG73	FMN, PLP, ZN, GAL, COA, ADP, SAM, FRU, NAD
PinR_V160A	ARG9, ILE10, SER11, GLN15, THR16, ASP72, ARG73	MG, ZN, GAL, COA, SAM, ADP, ARA, ADX, NAD
PinR_R3Q/V160A	ARG9, ILE10, SER11, THR12, GLU39, HIS40, ILE41, SER42, ALA46, THR47, SER48, ARG50, PRO51, ASP72, ARG73, LEU74, GLY75, CYS76, ASN77.	COA, GAL, NDP, MG, FMN, ADP, NAD

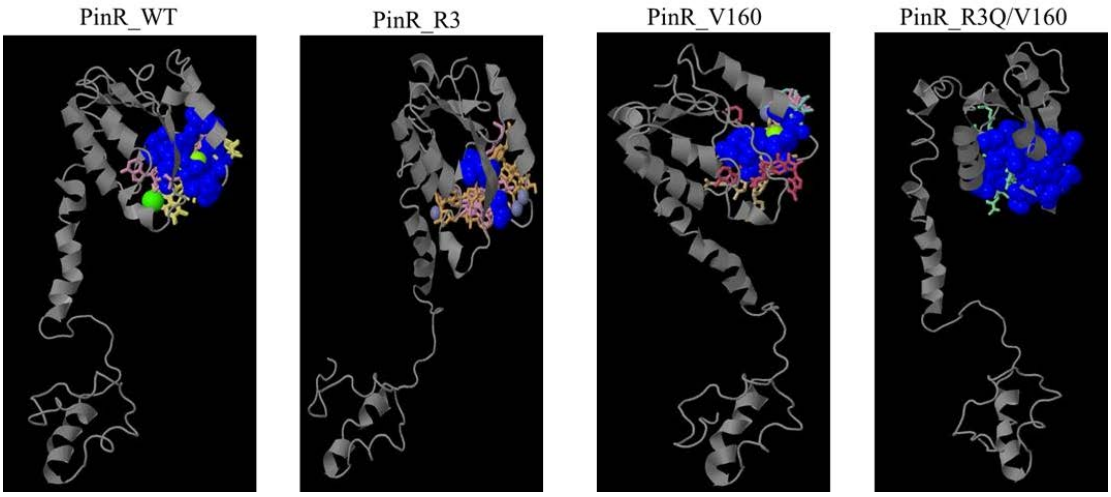


Figure 5.4. 3DLigandSite Models for the predicted structure of PinR. *PinR_WT, pinR_R3Q, pinR_V160A, and pinR_R3Q/V160A proteins are shown in grey with potential metallic heterogens shown as space fill and non-metallic heterogens as wireframe. Residues involved in bindings are blue. The mutation (s) of pinR likely affect the protein's active site, thereby possibly modifying the activity of pinR.*

5.2.3 Exposure of ampicillin fails to induce the emergence of resistance in *pinR* deletion strains

Now that I found gene mutations only occurred on the gene *pinR,* I would like to know what the role of *pinR* is in the emergence of antibiotic resistance induced by exposure of ampicillin. To test it, I directly knocked out the gene *pinR* on the basis of wild type or *ΔrecA* strains. I treated the *E. coli* strains with a single exposure of ampicillin at 50 μg/ml for 4 and 8 hours, and a cyclic treatment for continuous three weeks with a slightly modified protocol (Fig. 5.5), respectively. I found that that deletion of *recA* could be induced to be resistant, but deletion of *pinR* or double deletion of *pinR* and *recA* failed to be induced (Fig. 5.6A). Long-term exposure of ampicillin was able to induce the

evolution of resistance in wild-type and *ΔrecA* strain but not in *ΔpinR* and *ΔrecA*/*ΔpinR* strains (Fig. 5.6B), which together imply that *pinR* or its mutant protein may play a decisive role in the bacterial evolution of resistance.

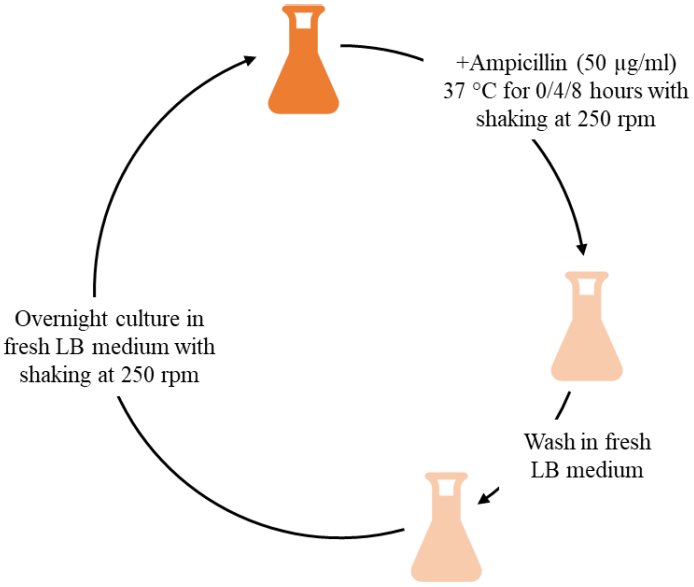


Figure 5.5 Evolutionary protocol of cyclic antibiotic exposure. *The experimental evolution protocol consists of three steps: 1) Killing. An overnight bacterial culture (~10^9 counts/ml) is diluted 1:100 into 50 ml LB medium with an addition of ampicillin at 50 μg/ml for 0m 4, and 8 hours incubation at 37 ºC with shaking at 250 rpm. 2) Washing. LB medium containing antibiotics are removed by washing twice in LB medium (centrifugation at 1,500×g for 20 min). 3) Growth. Survived bacteria is resuspended in 50 ml fresh LB medium and regrown overnight for 18 hours at 37 ºC with shaking at 250 rpm. Regrown culture is then repeated for the ampicillin killing for next cycle.*

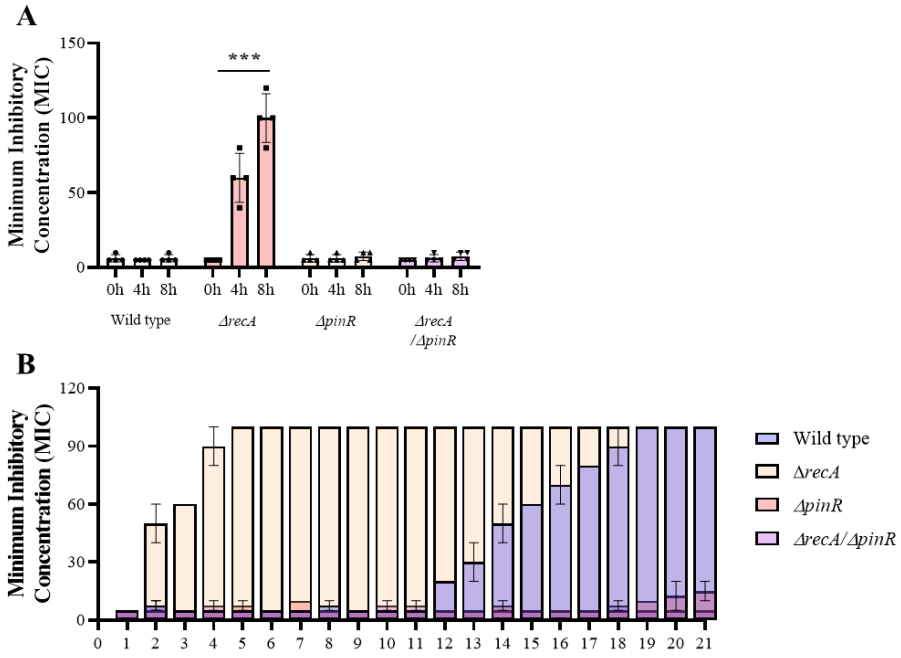


Figure 5.6 Exposure of ampicillin fails to induce resistance in pinR deletion strains. *(A) MICs value of evolved strains after a single exposure of ampicillin. (B) MICs value of evolved strains after a cyclic exposures of ampicillin for three weeks. X-axis represents the treating days. The data from (A) are presented as the mean ± s.d. of four independent experiments. The data from (B) are presented as the mean ± s.d. of two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. Mann-Whitney rank sum test.*

5.2.4 Single exposure of ampicillin did not change the copy number variations of β-lactamase associated genes

As described in Chapter 5.1.3 gene duplication and amplification which results in higher copy numbers of resistant genes is believed to contribute in the emergence of antibiotic resistance. Therefore, I further used the droplet digital PCR (ddPCR) to measure the copy number variations (CNVs) of *ampC*, *ampD*, *ampE*, *ampG*. Results show that even though

in the resistant isolates the level of β-lactamase is highly increased, I did not find any difference in *ΔrecA* resistant cells compared with other sensitive strains including wild type, overexpression of *recA*, *recA* restoring, *ΔpinR*, and *ΔpinR/ΔrecA* strains (Fig. 5.7). These results may indicate that the single exposure of ampicillin induced bacterial evolution of resistance is not associated with the elevated levels of DNA copy numbers.

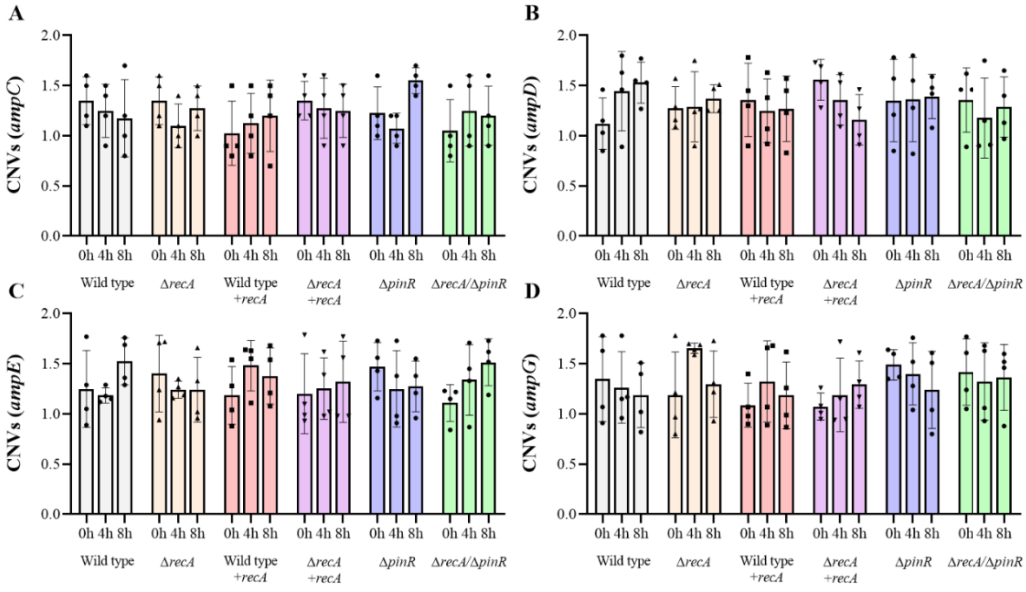


Figure 5.7. Copy number variations of β-lactamases associated genes. *All strains β-lactamases associated genes including ampC, ampD, ampE, and ampG (A-D) were detected using ddPCR measurements. All data are presented as the mean ± s.d. of four independent experiments.*

5.2.5 Single exposure of ampicillin increases the mRNA level of *ampC* both in resistant isolates and their daughter cells

In addition to increased DNA copy numbers, the expression of β-lactamases is regulated by transcriptional factors including mRNA of *ampC.* It is of significance that I quantified the expression of mRNA levels of *ampC* and found that its mRNA level was increased up to 2.3-fold in *ΔrecA* strain (Fig. 5.8A), indicating an unexpected mechanism for upregulation of the mRNA of *ampC* which is not resulted from the gene mutation. Prokaryotic bacterial mRNA is not stable, but I found that the mRNA expression of *ampC* was kept at a high level in their daughter cells cultured in a fresh medium for continues 7 days (Fig. 5.8B).

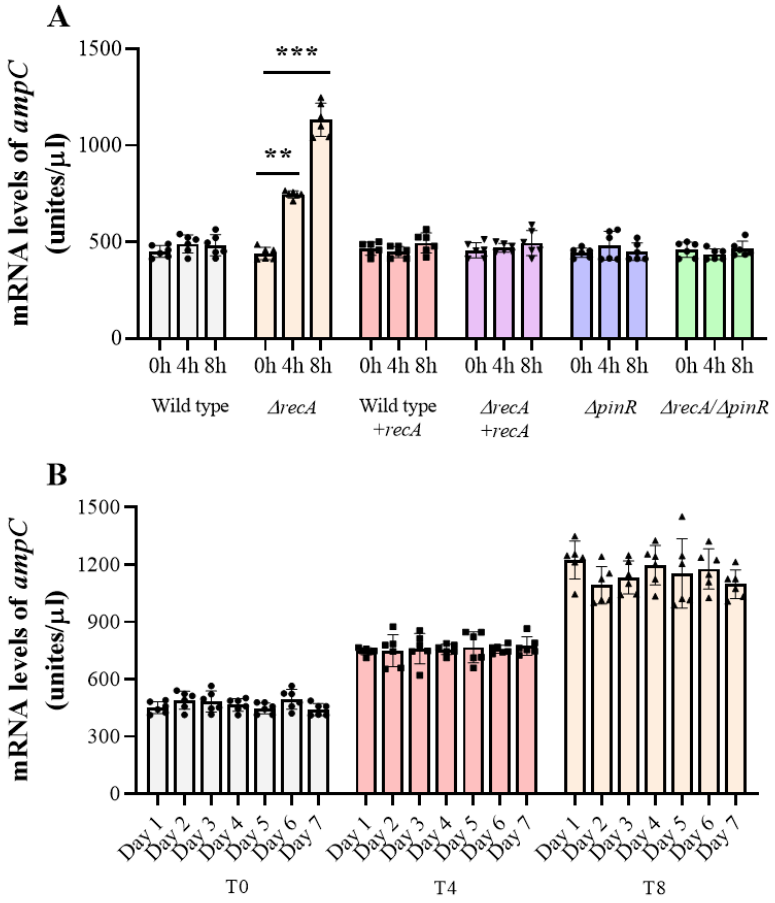


Figure 5.8 mRNA levels of gene ampC. *(A) mRNA levels of gene ampC in all E. coli strains after a single exposure of ampicillin at 50 μg/ml for 0, 4 and 8 hours, respectively. (B) mRNA levels of gene ampC in ΔrecA ancestral and resistant isolates and their daughter cells. All data are presented as the mean ± s.d. of 6 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. Mann-Whitney rank sum test.*

I measured other β-lactamase associated genes mRNA expression including *ampD, ampE,* and *ampG*, and found that all of their mRNA levels were not changed after the exposure of ampicillin in all *E. coli* strains (Fig 5.9), which demonstrated that the increased expression of β-lactamase was mediated by the upregulation of *ampC* mRNA.

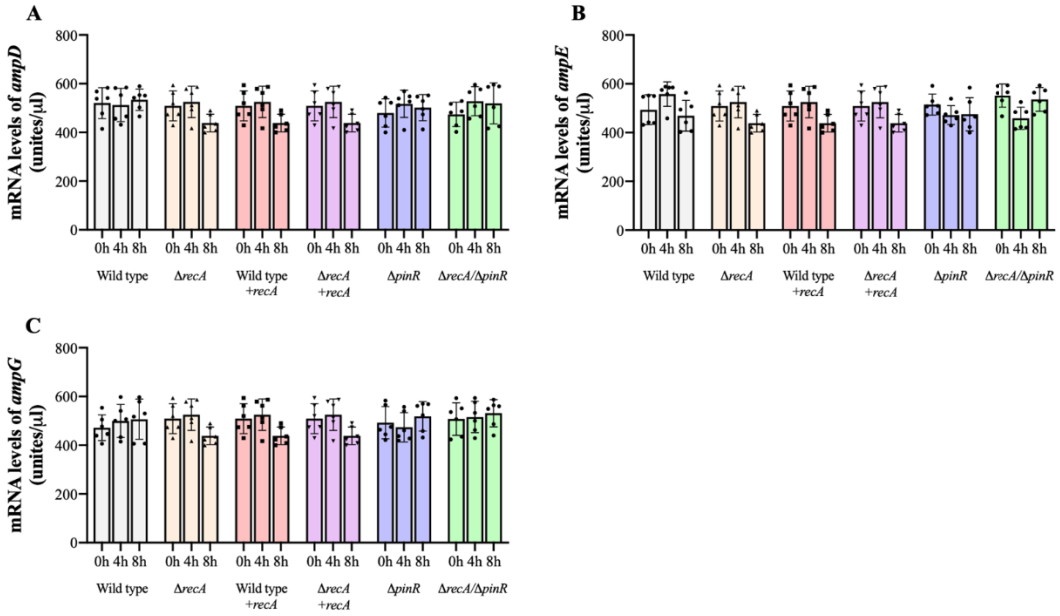


Figure 5.9 Bacterial mRNA levels. *mRNA levels of (A) ampD, (B) apmE, and (C) ampG in all E. coli strains after a single exposure of ampicillin at 50 μg/ml for 0, 4 and 8 hours, respectively. All data are presented as the mean ± s.e.m. from four individual experiments.*

5.3 Conclusion

The research in this chapter was performed to initiate the investigation into what the role of gene *pinR* in the bacterial evolution of resistance to antibiotics induced by the exposure of β-lactams antibiotics.

I explored the whole genome sequencing in all *E. coli* strains and found that gene mutations occurring on an uncharacterized gene *pinR*, which was further demonstrated to play a decisive role in the evolution of resistance, in which either short-term or long-term exposure of antibiotics failed to induce the evolution of resistance in *pinR* deletion strains.

I also found that the mRNA levels of *ampC* were highly increased both in resistance isolates and their following daughter cells. However, the DNA copy numbers were not changed among all β-lactamases associated genes.

These results demonstrate that the evolution of resistance is resulted from the increased mRNA expression of *ampC*, which turns to upregulate the expression of β-lactamases.

5.4 Reference

1. Davies, J. E. 1997. Origins, acquisition and dissemination of antibiotic resistance determinants. Ciba Found. Symp. 207:15–27.
2. Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399–406.
3. Luria, S. E., and M. Delbru¨ck. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491–511.
4. Riesenfeld, C., M. Everett, L. J. Piddock, and B. G. Hall. 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrob. Agents Chemother. 41:2059–2060.
5. Alonso, A., E. Campanario, and J. L. Martinez. 1999. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in Pseudomonas aeruginosa. Microbiology 145:2857–2862
6. Ren, L., M. S. Rahman, and M. Z. Humayun. 1999. Escherichia coli cells exposed to streptomycin display a mutator phenotype. J. Bacteriol. 181: 1043–1044.
7. Nakamura, S., M. Nakamura, T. Kojima, and H. Yoshida. 1989. gyrA and gyrB mutations in quinolone-resistant strains of Escherichia coli. Antimicrob. Agents Chemother. 33:254–255.
8. Kohler, T., M. Michea-Hamzehpour, P. Plesiat, A. L. Kahr, and J. C. Pechere. 1997. Differential selection of multidrug efflux systems by quinolones in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 41:2540–2543.

9. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev. 2009 Oct;22(4):582-610.
10. Edlund, T., Grundstrom, T. & Normark, S. Isolation and characterization of DNA repetitions carrying the chromosomal β-lactamase gene of Escherichia coli K-12. Mol. Gen. Genet. 173, 115–125 (1979).
11. Edlund, T. & Normark, S. Recombination between short DNA homologies causes tandem duplication. Nature 292, 269–271 (1981).

Chapter 6. Emergence of transit resistance induced by exposure of ampicillin and bacterial ribosomal inhibitors

Chapter 6. Emergence of transit resistance induced by exposure of ampicillin and bacterial ribosomal inhibitors

6.1 Introduction

6.1.1 Bacterial transient resistance to antibiotics

As described in Chapter 1, bacterial resistance to antibiotics is classified into two types, including “inherited resistance” and “non-inherited resistance”, which is also commonly called transient resistance. In contrast to inherited resistance resulting from mutations in existing genes or the organism’s acquisition on external resistance-encoding genes, non-inherited resistance is purely phenotypic. Non-inherited resistance can allow bacterial cells to temporarily survive under the lethal exposure of antibiotics, which is currently regarded through a stochastic mechanism where individual cells within a population display diverse phenotypes to hedge against the appearance of an antibiotic.

For example, a study in *Escherichia coli* showed that stress-induced variability within an isogenic population is transmissible between generations and plays a role in antibiotic survival at the single cell level, possibly by modifying membrane permeability [1]. Furthermore, cell populations can differentiate into resistant subpopulations with variable growth statuses due to cephalosporin hydrolase expression [2]. In *Salmonella enterica*, heterogeneous levels of porins and efflux pumps contribute to differential levels of antibiotic resistance [3]. In addition, single cell studies have shown that the probability of *E. coli* cell lysis correlates with the time since the last cell division [4]. Asymmetric cell

division events [5] and stochastic pulses in the catalase-peroxidase KatG [6] in mycobacteria result in differences in antibiotic susceptibility.

In addition to stochastic effects, cells can evade antibiotics by transiently inducing antibiotic resistance at the population level [7, 8]. A well-studied example of this is expression of protein MarA (the multiple antibiotic resistance activator), which plays a key role in multidrug resistance in enteric bacteria [9]. In *E. coli*, MarA expression can be induced by the addition of extracellular compounds, including antibiotics [10-12]. Thus, when antibiotics are detected, resistance genes are turned on, leading to population-wide resistance.

6.1.2 Chapter aims and objectives

The overall aim of this chapter was to understand the role of ribosome inhibitors during the evolution of resistance induced by ampicillin exposure. In particular, I would like to investigate if the exposure of ribosome inhibitors, chloramphenicol, could promote or suppress the evolution of resistance induced by ampicillin treatment.

6.2 Results

6.2.1 Ribosome inhibitors promote the emergence of transient resistance

I first exposed batch cultures of the wild type bacterium *E. coli* MG1655 to a high concentration of ampicillin at 50 μg/ml which is 10 times greater than the MIC but comparable to therapeutic doses for 0, 4, and 8 hours respectively (Fig. 6.1). After the

incubation, the ampicillin-containing medium was removed by washing twice. Survived isolates were then resuspended in LB medium with or without containing 6.25 µg/ml chloramphenicol (1/10 × MIC) for overnight culture. After the overnight culture, antibiotics were removed by washing twice with fresh LB medium, and cultures were directly analysed or stored at -80ºC.

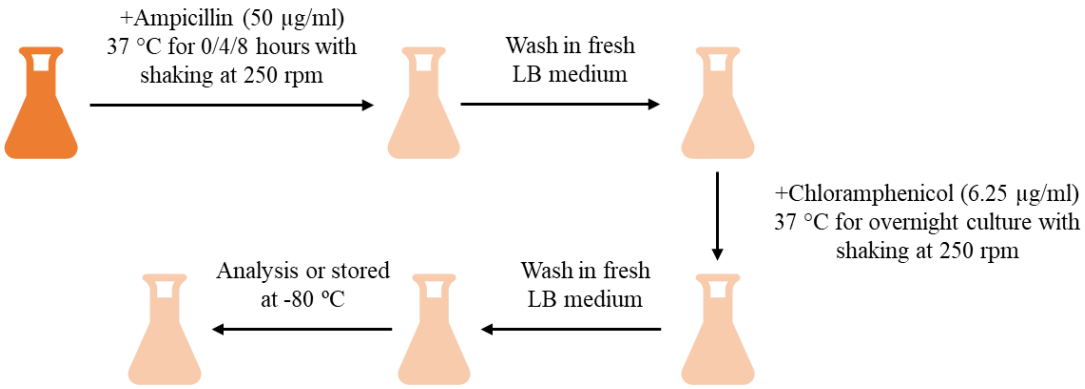


Figure 6.1 Evolutionary protocol of antibiotic exposure. *The experimental evolution protocol consists of four steps: 1) Killing. An overnight bacterial culture (~10^9 counts/ml) is diluted 1:100 into 50 ml LB medium with an addition of ampicillin at 50 µg/ml for 0m 4, and 8 hours incubation at 37 ºC with shaking at 250 rpm. 2) Washing. LB medium containing antibiotics are removed by washing twice in LB medium (centrifugation at 1,500×g for 20 min). 3) Inhibition. Survived isolates are resuspended in 30 ml fresh LB medium or LB medium containing 6.25 µg/ml chloramphenicol and grown overnight for 18 hours at 37 ºC with shaking at 250 rpm. 4) Secondary washing. LB medium containing antibiotics are removed by washing twice in LB medium (centrifugation at 1,500×g for 20 min). Regrown culture is then used for analysis or stored at -80 ºC.*

I found that with after the exposure of ampicillin for 8 hours, sub-treatment of chloramphenicol was able to induce the evolution of resistance but not for the strains under 4 hours treatment of ampicillin (Fig. 6.2A). However, without the treatment of chloramphenicol, single exposure of ampicillin could not trigger the emergence of resistance to β-lactams antibiotics (Fig. 6.2A). I further tested if this resistance is inheritable or not and found that after another overnight culture in fresh LB medium, all *E. coli* strains MICs resumed back to normal level (< 10 μg/ml), indicating that this resistance is a transient resistance (6.2B).

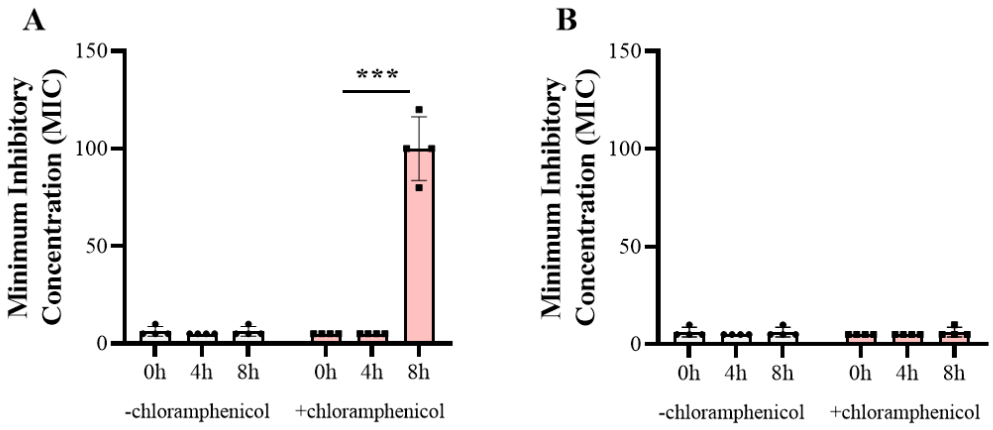


Figure 6.2 Resistance of the evolved strains. *(A) Increase in resistance of the evolved strains after the exposure of ampicillin and sub-treatment of chloramphenicol. Control groups were treated with fresh medium without containing chloramphenicol. (B) Transient resistance of the evolved strains. All evolved strains were regrown in fresh medium overnight. MIC was determined by measuring the minimum inhibitory concentration of ampicillin. All data are presented as the mean ± s.d. of six independent experiments. *p < 0.05; **p < 0.01; ***p < 0.00. Mann-Whitney rank sum test.*

6.2.2 Time-dependent evolution of resistance

I next compared the effects of timepoints of the chloramphenicol treatment for the survived isolates after the ampicillin exposure. It is interesting that, as found in chapter 6.1, survived isolates after the ampicillin exposure could be triggered to be resistance if it was immediately subtreated with the chloramphenicol; however, subtreatment of chloramphenicol failed to trigger the survived isolates to be resistance if the survived isolates regrown in fresh LB medium first (Fig. 6.3), suggesting that there is a time-dependent mechanism for the emergence of resistance induced by subtreatment of chloramphenicol.

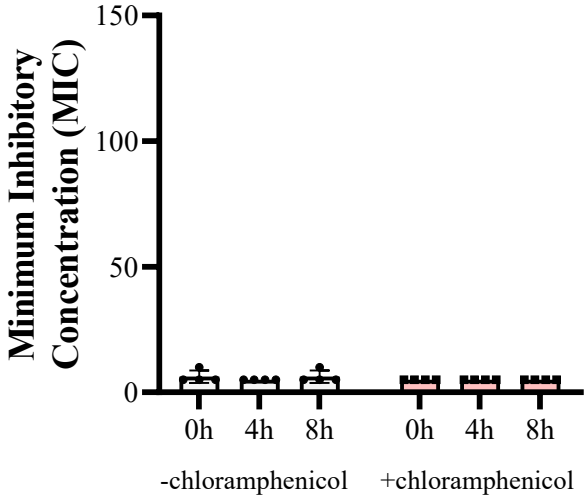


Figure 6.3 Time-dependent evolution of resistance. *After the exposure of ampicillin, all E. coli strains were regrown in fresh LB medium. Chloramphenicol were then added into the population for an overnight incubation. MIC was determined by measuring the minimum inhibitory concentration of ampicillin. All data are presented as the mean ± s.d. of six independent experiments.*

6.2.3 Level of β-lactamase is dramatically elevated in resistant isolates

I measured the expression of β-lactamase in culture medium of each strain after the exposures of ampicillin and chloramphenicol. Results showed that the expression of β-lactamase was significantly increased in *E. coli* strains after 8 hours exposure ampicillin and a following overnight incubation with chloramphenicol, which was similar with the emergence of resistance (Fig. 6.4A).

I also measured the level of β-lactamases in their daughter cells culture medium and found that the expression of β-lactamases resumed back normal level in all daughter cells (Fig. 6.4B), which may account for the reason why bacterial resistance to ampicillin was transient.

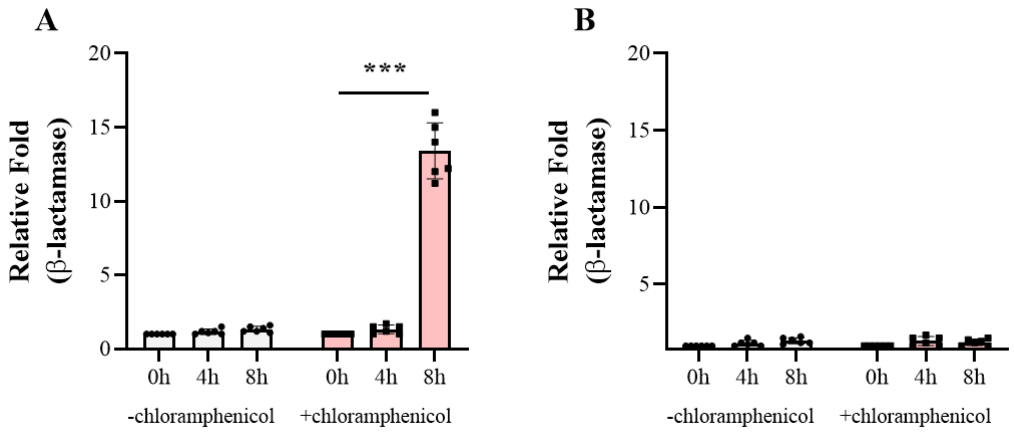


Figure 6.4 Increased level of β-lactamase in resistant isolates culture medium. *(A) Increased level of β-lactamase of the evolved strains after the exposure of ampicillin and sub-treatment of chloramphenicol. Control groups were treated with fresh medium*

*without containing chloramphenicol. (B) Level of β-lactamase of the evolved strains. All evolved strains were regrown in fresh medium overnight. All data are presented as the mean ± s.d. of six independent experiments. *p < 0.05; **p < 0.01; ***p < 0.00. Mann-Whitney rank sum test.*

6.3 Conclusions

The aim at this chapter was to study the role of subtreatment of bacterial ribosome inhibitors in the bacterial evolution of resistance to antibiotics induced after the exposure of β-lactams antibiotics.

I found that the subtreatment of chloramphenicol could only induce the emergence of resistance in *E. coli* strains which were exposed by ampicillin for 8 hours, but not for 4 hours exposure. This finding may suggest that the exposure of ampicillin in wild type *E. coli* may induce two types of tolerant cells.

The resistance I found in this chapter was not heritable, as its MIC value would be back to normal level in their daughter cells, suggesting that the subtreatment of chloramphenicol could trigger transient resistance in wild type *E. coli* strain which is different with previous findings in *ΔrecA* strain, even though the mechanism is not clear yet now.

6.4 Reference

1. Ni, M. et al. Pre-disposition and epigenetics govern variation in bacterial survival upon stress. PLoS genetics 8, e1003148,
2. Wang, X. et al. Heteroresistance at the single-cell level: adapting to antibiotic stress through a population-based strategy and growthcontrolled interphenotypic coordination. mBio 5, e00942–00913.
3. Sanchez-Romero, M. A. & Casadesus, J. Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. Proceedings of the National Academy of Sciences of the United States of America 111, 355–360.
4. Lambert, G. & Kussell, E. Quantifying Selective Pressures Driving Bacterial Evolution Using Lineage Analysis. Physical Review 5, 2160–3308.
5. Aldridge, B. B. et al. Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. Science 335, 100–104 (2012).
6. Wakamoto, Y. et al. Dynamic persistence of antibiotic-stressed mycobacteria. Science 339, 91–95.
7. Nikaido, H. Multidrug resistance in bacteria. Annual review of biochemistry 78, 119–146.
8. Piddock, L. J. Understanding the basis of antibiotic resistance: a platform for drug discovery. Microbiology 160, 2366–2373
9. Ruiz, C. & Levy, S. B. Many chromosomal genes modulate MarA-mediated multidrug resistance in Escherichia coli. Antimicrobial agents and chemotherapy 54, 2125–2134.
10. Cohen, S. P., Levy, S. B., Foulds, J. & Rosner, J. L. Salicylate induction of antibiotic resistance in Escherichia coli: activation of the mar operon and a mar-independent pathway. Journal of bacteriology 175, 7856–7862 (1993).
11. Martin, R. G. & Rosner, J. L. Transcriptional and translational regulation of the marRAB multiple antibiotic resistance operon in Escherichia coli. Molecular microbiology 53, 183–191.
12. Hachler, H., Cohen, S. P. & Levy, S. B. marA, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in Escherichia coli. Journal of bacteriology 173, 5532–5538 (1991).

Chapter 7. Discussion and perspective

Chapter 7. Discussion and perspective

7.1 Superfast evolution of antibiotic resistance

The failure of antibiotics treatment typically results in the emergence of resistance which has been a globally growing problem. Many studies show that sub-lethal concentration of antibiotic exposure or transit antibiotic treatment can induce the formation of persistence or tolerance. Whereas, cyclic exposure (usually 8-17 days) of antibiotics is demonstrated to promote the accumulation of bacterial adaptive mutations in tolerance which in turn pave the way for subsequent evolution of resistance.

Notably, the induction of SOS response is always believed to aid bacterial propagation defence against antibiotic lethality which requires the SOS-promoting *recA* and *lexA* genes which are regarded as potential drug targets attacking process of SOS response (Fig. 7.1A). I here reported that in the presence of *recA*, single exposure of β-lactam antibiotics can trigger the emergence of tolerance through the induction of SOS response (Fig. 7.1B); however, once the *recA* is deleted, it causes a superfast evolution (within 8 hours treatment) of resistance which requires new identified gene mutations occurring on an uncharacterized gene *pinR* but not dependent on the induction of SOS response. (Fig. 7.1C).

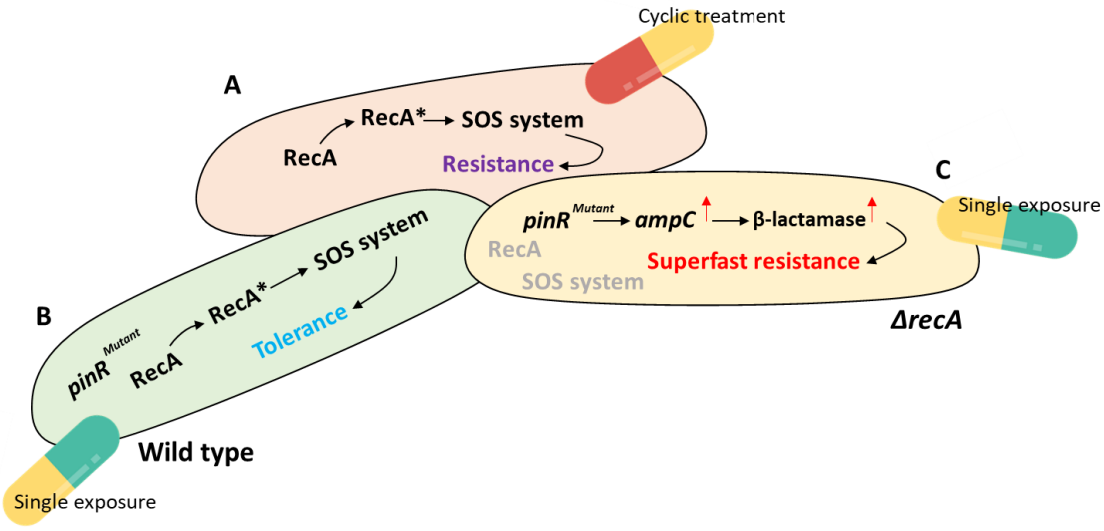


Figure 7.1 Models for bacterial evolution of resistance. *(A) In wild type strain, exposure of certain antibiotics causes the activation of RecA, induction of SOS response and adaptive gene mutations which can be stimulated in cells underwent subsequent cyclic treatment (usually 8 -17 days) resulting in the evolution of resistance. I report here that (B) a single exposure of β-lactam antibiotics can trigger the mutations on gene pinR, but in the presence of recA cells are evolved to be tolerance through the induction of SOS response; however (C) in the absence of recA, pinR mutation may lead to a superfast evolution of resistance in ΔrecA strain in which the mRNA level of ampC is significantly upregulated resulting in higher expression of β-lactamase.*

The link between bactericidal antibiotic activity, activation of the SOS response, and induction of antibiotic resistance demonstrates the potential for reducing resistance by targeting proteins essential for SOS response. Bactericidal antibiotic-mediated DNA damage results in the formation of RecA-ssDNA filaments which are key intermediates in DNA repair mechanisms and the SOS response. The repair of antibiotic-induced DNA damage by the SOS response has the unintended effect of promoting the formation of a

hypermutable state that promotes mutagenesis and transmission of antibiotic resistance genes. Therefore, suppression or attenuation of the SOS response system via preferential inhibition of RecA is proposed as a possible therapeutic strategy to suppress the development of antibiotic multiresistance and resistance-conferring mutagenesis.

However, this opinion has been challenged in this thesis, because I find deficiency of *recA* promotes a superfast evolution of bacterial resistance to β-lactams antibiotics (Chapter 3). More importantly, this resistance I observed is heritable. In some cases, single exposure of antibiotics is reported to induce transient resistance which is phenotypic resistance as when the antibiotic is removed the resistant isolates are sensitive to antibiotics. This finding first demonstrates that the development of bacterial drugs targeting RecA is not applicable when it is designed to be used combined with β-lactam antibiotics.

7.2 PinR may be a new drug target

This study revealed PinR as a potential pharmacologic target in several aspects. First, *pinR* deletion on the basis of *recA* deletion resulted in a 20-fold reduction in the ampicillin MIC after a single exposure of ampicillin. Using the same dose of ampicillin would result in a significant delay of the resistant establishment in *ΔpinR* strain compared with the wild type and *ΔrecA* strains under the cyclic treatment (Chapter 5).

Second, I also attempted to explore if there was any additional benefit of *pinR* deletion after normalizing drug exposure to MIC. Our results suggested that *pinR* deletion delayed the onset of resistance emergence in *E. coli* apart from conferring a reduction in the MIC.

Third, I observed a marked decreased expression of β-lactamase in *pinR* deletion cells which mainly contributes to the emergence fo resistance to β-lactam. Keeping in mind this decrease in the bacterial burden and delay in the onset of resistance, a “hit hard and early” approach could be applied to maximize the benefit of PinR inhibition.

Finally, from the molecular aspect, since no preexisting mutants were expected to be present at baseline, it was believed that isolates with diverse mutation on *pinR* will casue the evolution of resistance. Resistance development was most favoured and likely to be observed with a low to intermediate drug exposure. Thus, these findings open the door to investigate the role of PinR as a novel antimicrobial target for drug development.

7.3 Regulation of mRNA level of *ampC*

The mechanisms of how bacterial DNA recombinases regulate the evolution of resistance to β-lactam antibiotics is not fully clear yet, but I find that it seems to be associated with the increased mRNA levels of *ampC*. The heritable resistance is supposed to be resulted from an unexpected synergistic effect, first, in the presence of antibiotics, deficiency of *recA* and mutations on *pinR* promote the higher expression of *ampC* mRNA; secondly, after the promotion, resistant isolates *ampC* mRNA is able to be continuously upregulated even in the absence of antibiotics.

However, there is a question remaining about the higher expression of *ampC* mRNA can be maintained in resistant isolates when the antibiotic is removed. I have two hypothesises to account for this phenomenon.

First, PinR is predicted to belong DNA resolvase family sharing a similar similar structure with the protein of dna-invertase and serine recombinase. Mutations on *pinR* are predicted to cause the charge of the local environment to affect the catalytic activity of PinR, and increase the efficiency of DNA binding activation. Thus, mutated PinR may result in several other genes DNA copy number variations despite what I measured in this thesis including *ampC, ampD, ampE,* ang *ampG*. On the other hand, gene transcription of *ampC* is regulated by several DNA binding transcriptional dual regulators, for example regulator *nac*, *bolA*, or OxyR. Therefore, it is possible that these DNA binding transcriptional regulators further influence the transcription of *ampC* in resistant isolates when the antibiotic is removed.

Second, bacterial DNA methylations may contribute to this process. DNA methylation is a process by which methyl groups are added to the DNA molecule. Methylation can change the activity of a DNA segment without changing the sequence. When located in a gene promoter, DNA methylation can act to promotes gene transcription. Bacterial genomes contain small amounts of N_4-methylcytosine, C_5-methylcytosine, and N_6-methyladenine. Base methylation is catalysed by DNA methyltransferases that recognize specific DNA motifs, and it occurs after DNA replication. For I do not observe the change of gene sequence apart from *pinR*, I hypothesize that this higher expression of *ampC* may be resulted from uncharacterized DNA methylation in resistant isolate which can be inherited to daughter cells.

These two hypothesis will be investigate in future in order to understand the mechanism for superfast evolution of resistance to β-lactams antibiotics in *E. coli* strains with *recA* deletion.

7.4 Perspectives

This work introduces the superfast evolution of antibiotic resistance to β-lactam antibiotics mediated by DNA recombination. PinR as a new identified protein involved in the evolution of resistance needs to be carefully investigated in future including its structure, function and cellular laicization.

The second target for future study is to understand the interaction between RecA and PinR, both serving as DNA recombinases. Moreover, I showed that PinR may contribute in the emergence of resistance to β-lactam antibiotics, but there is no evidence demonstrating if it shares a similar function to other classes of antibiotics.

Third, as discussed above, whether bacterial DNA methylations contributes to the emergence of resistance is not clear yet now, even though I found the mRNA expression could be maintained in resistant daughter cells for seven days.

Appendix

Paper: Superfast bacterial evolution of resistance to β-lactam antibiotics mediated by bacterial DNA recombinases

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Superfast evolution of antibiotic resistance

Exposure of antibiotic can induce bacterial mutagenesis and trigger the evolution of resistance. Recent studies show that SOS response plays a critical role aiding bacterial propagation defence against antibiotic lethality which requires the activation of DNA recombinase RecA. Le *et al.* found that, however, a single exposure of β-lactam antibiotics triggered a superfast evolution of heritable cross-resistance in *recA* deletion *E. coli* strain within 8 hours. Moreover, an uncharacterized gene *pinR* was first identified to play a decisive role in the evolution of resistance potentially acting as a DNA recombinase. Inhibition of PinR delayed the evolution of antibiotic resistance suggesting that PinR may serve as a promising novel target for future drug development.

Abstract

RecA has been considered as a drug target to suppress the induction of SOS response towards the evolution of resistance induced by the broad-spectrum fluoroquinolone antibiotics. Here, we report that single exposure of β-lactam antibiotics can trigger a superfast evolution of resistance in *recA* deletion *E. coli* strain, independent to the SOS response. This type of single exposure causes gene mutations on an uncharacterized gene *pinR*, and its encoding protein may be involved in DNA recombination. Moreover, single or intermittent treatment of β-lactam antibiotics fails to induce the resistance in *pinR* deletion *E. coli* strain. This work highlights the antagonistic role among DNA recombinases in the emergence of antibiotic resistance, and demonstrates that loss of *recA* increases the rate of resistance to β-lactam antibiotics, but PinR is likely to be a novel drug target.

The failure of antibiotics treatment typically results in the emergence of resistance as a globally growing problem [**1, 2**]. Many studies show that sub-lethal concentration of antibiotic exposure or transit antibiotic treatment can induce the formation of persistence or tolerance [**3, 4**]; whereas, cyclic exposure (usually 8-17 days) of antibiotics promote the accumulation of bacterial adaptive mutations in persisters towards the subsequent evolution of resistance [**5, 6**] (Fig. 1). However, the antibiotics induced mutagenesis that damage bacterial DNA has been poorly understood. Notably, the induction of SOS response has always been believed to aid bacterial propagation defence against antibiotic lethality, which requires the activation of SOS-promoting *recA* [**7-9**]. A recent study implicated the essential role of RecA in the evolution of resistance to fluoroquinolones antibiotics [**10**], but it is unclear whether this is a conserved mechanism of resistance in response to different antibiotic classes.

In our experiment, we first exposed a batch culture of *E. coli* to a single dose of ampicillin at 50 μg/ml (10 × MIC) that is a comparable dose to therapeutic and fixed residual level used in clinical for 0, 4, and 8 hours, respectively, (Fig S1A). Though the initial testing of the wild type strain did not result in resistance (Fig. 2A), capacity of tolerance, measured by the MDK_{99} and phenotypic distribution of the lag period and growth rate, suggested that wild type cells had evolved to be tolerance by lag-phase, which result was in agreement with the previous models of antibiotic resistance evolution (Fig. S2) [**3-5**].

In sharp contrast, when exposing *E. coli ΔrecA* to a single dose of ampicillin (50 μg/ml), we surprisingly found emergence of resistance (up to 20-fold increase in MIC) after only 8 hours of antibiotic exposure (Fig. 2A and B). This type of resistance in *ΔrecA* cells after a single exposure can be broadly induced by other β-lactam antibiotics, including penicillin G and carbenicillin (Fig. S3). Interesting, expression of exogenous *recA* increased the susceptibility of *ΔrecA* to ampicillin, whilst overexpression of *recA* did not affect the emergence of resistance (Fig. 2A and B). Determination of the mutant prevention concentration (MPC) demonstrated that a single exposure of ampicillin induced a higher mutation frequency in *ΔrecA* cells, compared with the wild type (from 10^{-9} to 10^{-7} mutations per generation, respectively), and this could be restored to wild type rate of mutation frequency by *recA* complementation (Fig. 2C). Furthermore, the observed ampicillin resistance in *ΔrecA* cells was stable and heritable, as cultures continuously grown in antibiotic-free medium were still resistant to ampicillin (10× MIC) after seven days (Fig. S4). Thus, in contrast to the reported fluoroquinolone antibiotic resistance, loss of RecA results in a superfast evolution of stable and heritable resistance to β-lactam antibiotics.

The evolution of resistance in *ΔrecA* cells appeared to be a conserved phenomenon in response to β-lactams; thereby we further investigated if resistance confers cross-resistance to different classes of antibiotics. We found that the resistant isolates induced by a single exposure to ampicillin exhibited cross-resistance to other β-lactams antibiotics (including penicillin G and carbenicillin) but were sensitive to other classes of antibiotics such as ciprofloxacin and chloramphenicol, suggesting that cross-resistance conferred by ampicillin-resistant *ΔrecA* cells is limited to β-lactams (Fig. S5).

Elevated expression of β-lactamase contributes to the emergence of resistance to β-lactam antibiotics [**11, 12**]. Indeed, we observed that the production of β-lactamase was significantly increased in the *ΔrecA* strain after a single exposure to ampicillin (Fig. 2D). More importantly, in parallel with the heritable resistance, we found that the production of β-lactamase was continuously increased even after continuous culture in antibiotic-free medium (Fig. S6), suggesting that the heritable resistance as a result of a consistent production of β-lactamase induced by the single exposure of β-lactam antibiotics. It was notable that

resistance could not be reverted by *recA* complementation once resistance had been established, since transformation of a *recA* expressing plasmid to *ΔrecA* resistant isolates had no effect on the MIC of ampicillin and β-lactamase expression (Fig S7A and B). This indicates that resistance is established prior to the expression of *recA*.

RecA is a pleiotropic recombinase involved in both homologous recombination, and during the SOS response. To test if the SOS response is involved in the emergence of resistance in the absence of RecA, we applied a dual-colour Stochastic Optical Reconstruction Microscopy (STORM) to pinpoint the dynamic structure of the *E.coli* chromosome and the distribution of SOS related error-prone DNA polymerase at a single cell level. We observed that exposure to ampicillin dramatically increased the length of the surviving bacteria both in wild type and *ΔrecA* strains, indicating inhibition of the division process and formation of filaments (Fig. 3A and B). In the absence of ampicillin exposure, the duplication and segregation of the chromosome could be observed in wild type and *ΔrecA* cells, as seen by the appearance of two distinct foci (0h shown in Fig. 3C and D), whilst in filaments multiple chromosomes were observed that undergo asynchronous replication or separation of foci (Fig. 3E) with a liner relation between filament length and chromosome number (Fig. 3F). These observations suggest that both tolerant (wild type) and resistant (*ΔrecA*) strains survive against ampicillin lethality through cell division arrest, which results in multinucleated filaments.

STORM further revealed that ampicillin exposure in wild type cells resulted in a dramatic increase in the co-localization ratio between the chromosome and DNA polymerase (18.7 ± 2.5% to 88.7 ± 5.6% after 8 hours ampicillin treatment), indicating an induction of SOS response (Fig. 3G and I). However, in the absence of *recA*, the co-localization ratio of only 57.6 ± 4.5% was achieved after 8 hours exposure (Fig. 3H and I), suggesting that the SOS response was less active after ampicillin treatment in *ΔrecA* cells.

We further used three mutants of the SOS response to determine their contributions to the emergence of resistance after ampicillin treatment: *ΔlexA*, resulting in constitutive induction of the SOS response; $lexA^{ind-}$, where SOS response is always switched off; and *ΔsulA*, where induction of SOS response is inhibited due to loss of the effector protein SulA. As depicted in Fig. 3J, none of *ΔlexA*, $lexA^{ind-}$ and *ΔsulA* strains exhibited resistance to ampicillin,

suggesting that the SOS response-specific role of RecA is not responsible for evolution of resistance.

Previous studies have shown that SNPs mutation within the coding region or promoter of *ampC* in transcription of β-lactamase could largely increase β-lactamase expression, providing high resistance to β-lactam antibiotics [**13, 14**]. We therefore performed the whole genome sequencing of resistant isolates, and surprisingly found that in either wild type or *ΔrecA* cells, no SNP mutations were present within the genes encoding for β-lactamase associated enzymes, including *ampC* and its regulation genes *ampD, ampE,* and *ampG* (Fig. 4A) [**15**]. But three SNPs in an uncharacterized gene named *pinR* were found in *ΔrecA* cells after ampicillin exposure, resulting in non-synonymous mutations (Fig. 4A). More interestingly, mutations within *pinR* could be also detected in wild type strain after a single ampicillin exposure (Fig. 4A). To further test the role of PinR in the emergence of resistance, we constructed *pinR* deletion mutants in both wild type and *ΔrecA* strains. We found that deletion of *pinR* or double deletion of *pinR* and *recA* failed to generate resistant cells after a single exposure of ampicillin (Figure 4B). For an intermittent experiment, we treated *E. coli* strains continually for three weeks (Fig. S1B), and found that long-term exposure to ampicillin induced the evolution of resistance in wild type and *ΔrecA* strain but not in *ΔpinR and ΔrecA/ΔpinR* strains (Fig. 4C). These results together suggest that *pinR* may play a decisive role in the bacterial evolution of resistance.

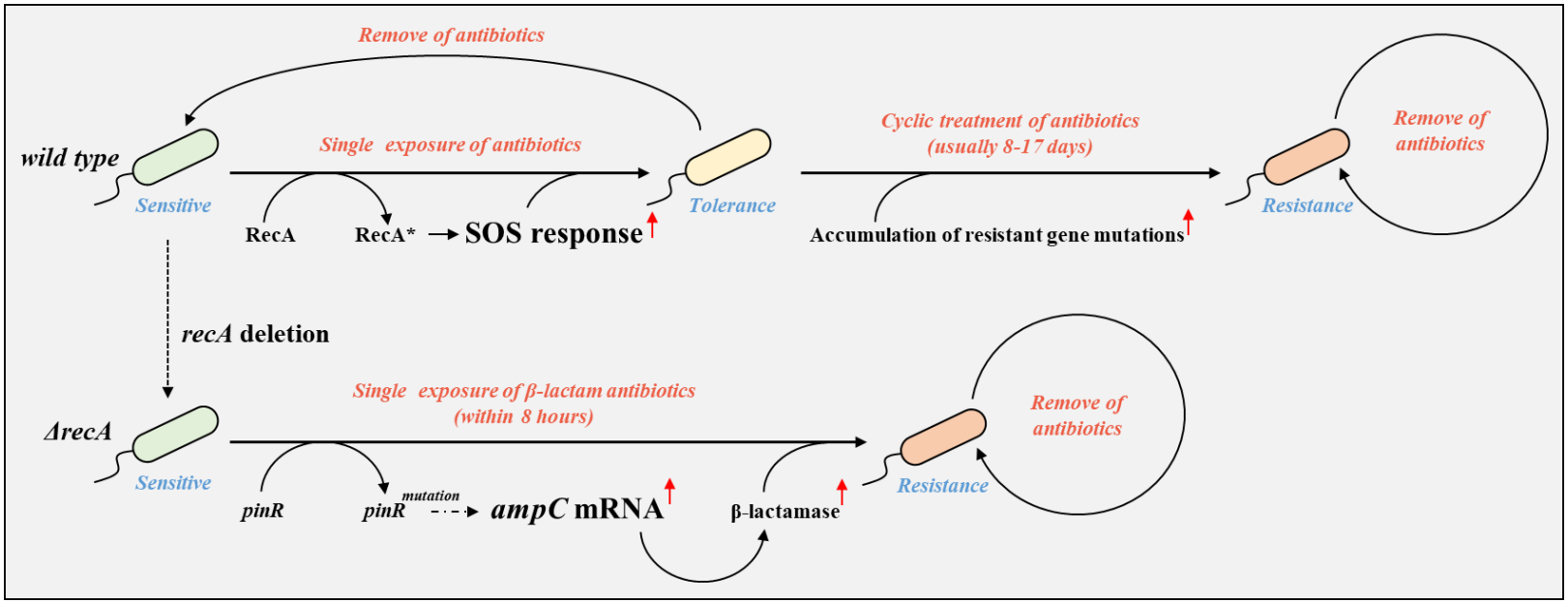
PinR is a protein with unknown structure and function that is encoded within the rac prophage [**16**]. Phyre[2] predicts that the protein PinR shares a similar structure with the protein of DNA-invertase and serine recombinase [**17**] (Fig. 4D), and gene mutations of *pinR* may affect the protein's local structure and overall function (Fig. S8 and Table S4). We did not detect genetic mutations within *ampC*, but previous studies showed that changes in β-lactamase expression could be detected via increased copy numbers of *ampC*, which was regulated through homologous recombination [**18-20**]. PinR was predicted to be a bacterial DNA recombinase, we therefore used droplet digital PCR (ddPCR) to measure the copy number variations (CNVs) of *ampC*, *ampD*, *ampE*, and *ampG* in *ΔrecA* resistant isolates and found that none of their CNVs were changed (Fig. S9A-D). This suggested that increased copy number is not responsible for the increased β-lactamase expression observed in our study (Fig. 2D). However, by further quantification of the mRNA levels of these genes, we

found that the mRNA level of *ampC* increased by up to 2.3-fold in *ΔrecA* strain (Fig. 4E and Fig S10 A-C). This result indicated an unexpected mechanism for elevated production of β-lactamase via the upregulation of mRNA level of *ampC*, but not resulted from the β-lactamase-associated gene mutations (Fig. 4A). Prokaryotic bacterial mRNA is not stable, but we found that the mRNA expression of *ampC* was maintained at a high level in *ΔrecA* resistant daughter cells cultured in a antibiotic-free medium for seven days (Fig. 4F), correlating with our observations of β-lactamase expression.

Altogether, these results broaden our knowledge on how bacterial DNA recombination mediates the evolution of resistance to antibiotics induced by exposure of β-lactam antibiotics. RecA is well-known to act as a critical pleiotropic recombinase, participating in bacterial SOS response and believed to be a universal drug target [**7, 21, 22**]. However, we here show that deficiency of *recA* causes an unexpected consequence in the superfast evolution of heritable resistance to β-lactam antibiotics. This process is in a SOS response-independent manner, suggesting that RecA plays a different role in response to different classes of antibiotics. More importantly, the cross-resistance to β-lactam antibiotics even without using them as the primary treatment is observed in *ΔrecA* resistant isolates, which implies that it may largely narrow our clinical option of antibiotics in future. Therefore, the development of antibacterial drug targeting RecA seems impracticable if they are designed to be used in combination with β-lactam antibiotics.

Evolution of heritable resistance to antibiotics is usually regarded as a consequence of accumulation of resistant gene mutations [**6**]. However, we here report a new mechanism for superfast evolution of heritable resistance, in particular, single exposure of β-lactam antibiotics upregulates the expression of mRNA of *ampC* but not induces gene mutations expect for *pinR* in *recA* deletion *E. coli* strain. Notably, this elevated expression of mRNA can be surprisingly maintained in cells cultured in antibiotic-free medium, even though mRNA is not expected to be continually maintained in the absence of a cue. This finding demonstrates that bacterial heritable resistance can also be caused by the change of transcriptions instead of gene mutations.

This is the first report to show that PinR may play an uncharacterized role as a bacterial DNA recombinase involved in the evolution of resistance to β-lactam antibiotics, because deletion of *pinR* dramatically delays the evolution of antibiotic resistance either exposed to a single dose of antibiotics in the absence of *recA*, or under intermittent treatment of antibiotics in the presence of *recA*. *pinR* is a global Rac phage gene expressed in many bacteria [**23**], thereby PinR may serve as a promising novel target for future drug development.



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200 **Figure 1. Models for bacterial evolution of resistance.** Exposure of antibiotics causes the activation of RecA, induction of SOS response and
201 adaptive gene mutations leading to the evolution of heritable resistance in wild type *E. coli* strain. Instead, a single exposure of β-lactam
202 antibiotics induces an uncharacterized gene *pinR* mutations, upregulation of the *ampC* mRNA expression resulting in a superfast evolution of
203 heritable resistance in *recA* deletion *E. coli* strain.

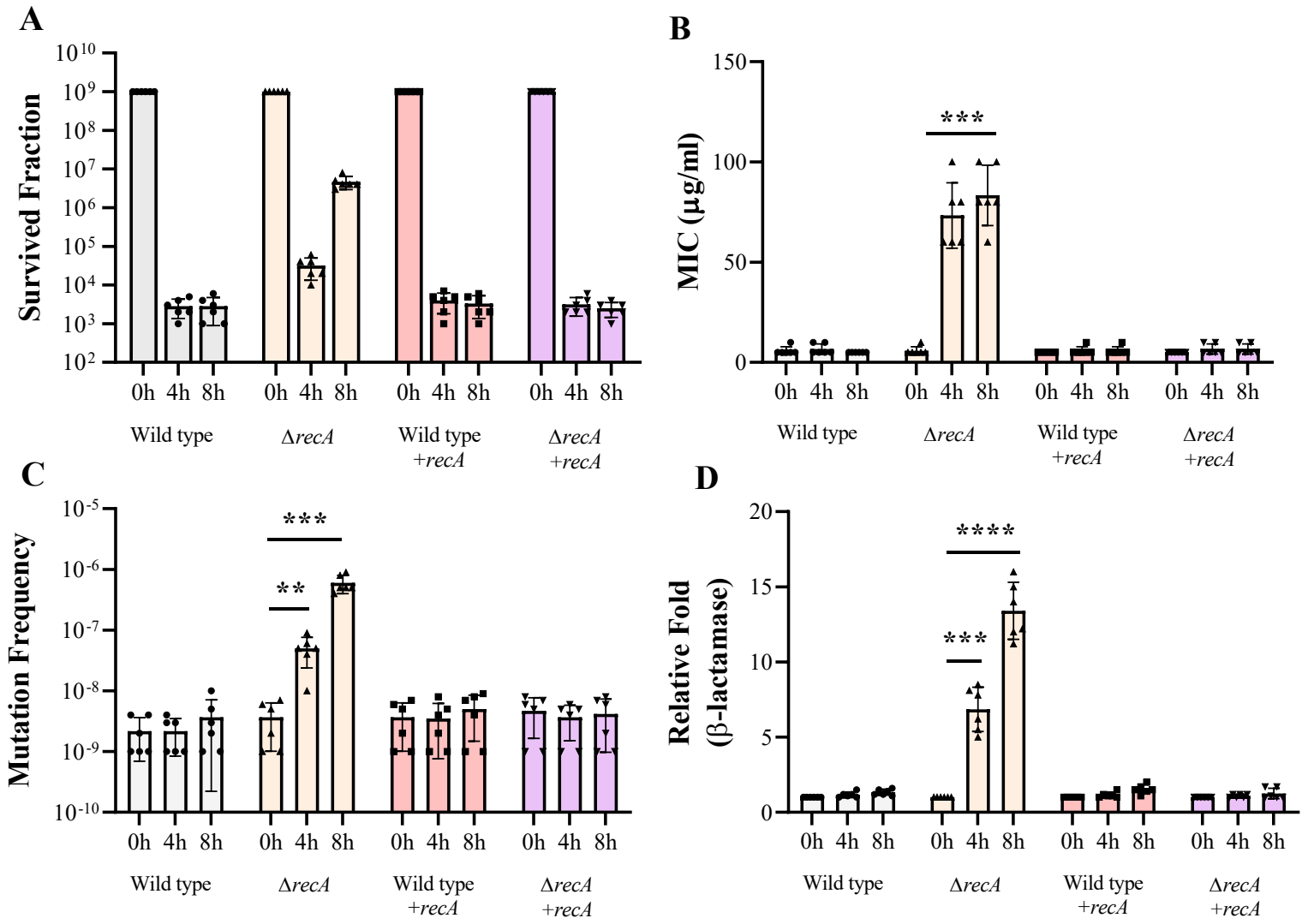
A
Survived Fraction
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
0h 4h 8h
Wild type
Δ*recA*
Wild type +*recA*
Δ*recA* +*recA*
B
MIC (μg/ml)
150
100
50
0
***
C
Mutation Frequency
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
**
***
D
Relative Fold (β-lactamase)
20
15
10
5
0
***
****

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Figure 2. *recA* deficiency promotes the bacterial evolution of resistance to β-lactam antibiotics. (A) Survival of all strains after 50 μg/ml ampicillin exposure for 0, 4, and 8 hours, respectively. (B) MICs of all strains after the ampicillin treatment for 0, 4, and 8 hours, respectively. (C) Mutation frequency in all *E. coli* strains after the treatment of ampicillin for 0, 4, and 8 hours, respectively. (D) β-lactamases levels in all strains after ampicillin exposure for 0, 4, and 8 hours, respectively. All data are presented as the mean ± s.d. of six independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Mann-Whitney rank sum test.

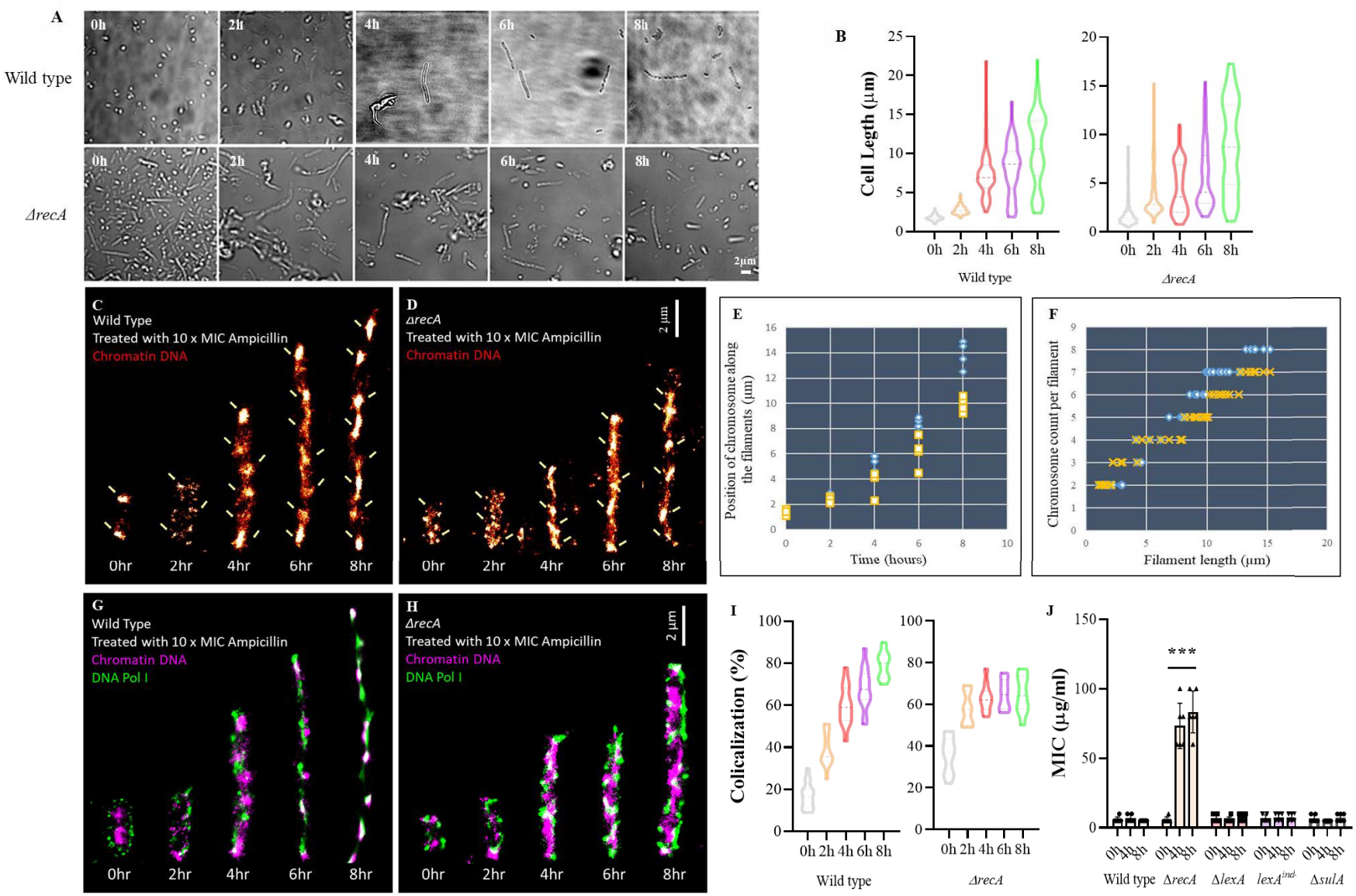
A
Wild type
ΔrecA
0h
2h
4h
6h
8h
2μm
B
Cell Legth (μm)
Wild type
ΔrecA
C
Wild Type
Treated with 10 x MIC Ampicillin
Chromatin DNA
D
ΔrecA
Treated with 10 x MIC Ampicillin
Chromatin DNA
2 μm
0hr
2hr
4hr
6hr
8hr
E
Position of chromosome along the filaments (μm)
Time (hours)
F
Chromosome count per filament
Filament length (μm)
G
Wild Type
Treated with 10 x MIC Ampicillin
Chromatin DNA
DNA Pol I
H
ΔrecA
Treated with 10 x MIC Ampicillin
Chromatin DNA
DNA Pol I
I
Colicalization (%)
Wild type
ΔrecA
J
MIC (μg/ml)
***
Wild type
ΔrecA
ΔlexA
lexA^ind-
ΔsulA

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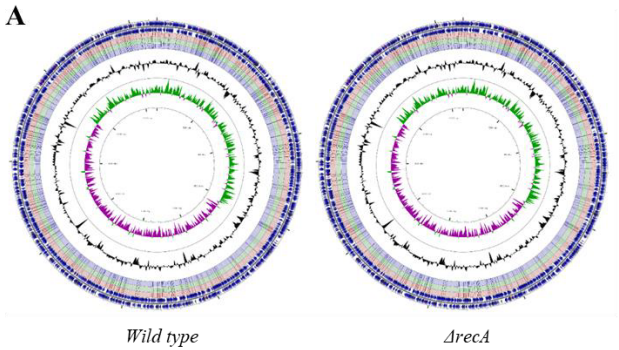
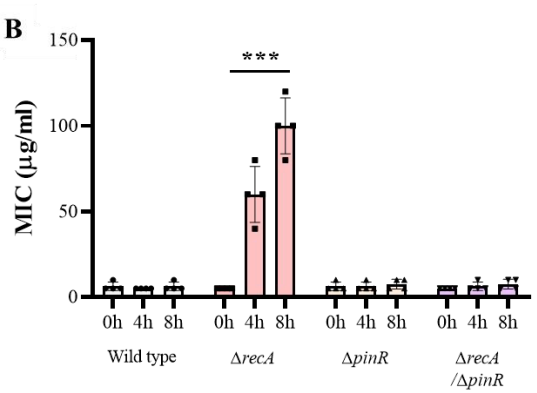
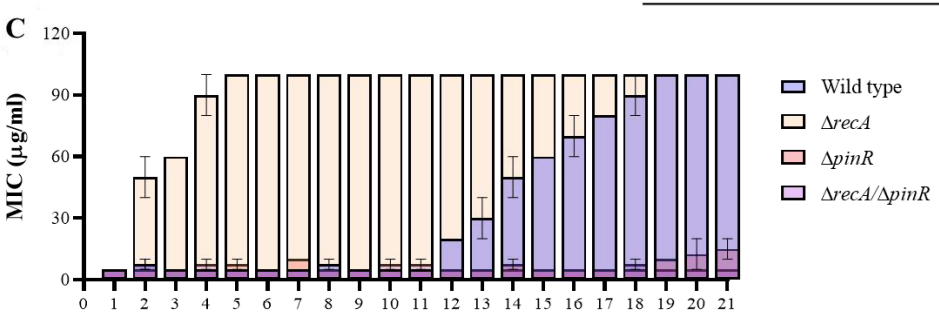
Figure 3. Induction of SOS response in the emergence of resistance. (A) After each killing for 0, 2, 4, 6, and 8 hours by exposure of ampicillin at 50 μg/ml, cells were immediately fixed to be imaged by TIRF microscopy. (B) Cell length counted in each group after different exposure time (ampicillin, 50 μg/ml). Each group contains bacterial number > 200. (C and D) STORM images reveal the formation of multinucleated filaments induced by exposure of ampicillin (10 × MIC) both in wild type and *ΔrecA* strains. Scar bar = 2 μm. Yellow arrow indicates single chromosome foci. (E). Distribution of chromosome loci positions in individual cells (Selected shown here, n = 4). (F) Chromosome count as a function of length. (Selected shown here, n = 6). (G and H) Dual color STORM images reveal a movement of DNA polymerase I from cell membrane to cytoplasm binding to DNA. Scarle bar = 2 μm. Each group contains bacterial cell number > 200. (I) Statistical calculation of the co-localization between bacterial chromosome and DNA polymerase I. Scarle bar = 2 μm. Each group contains bacterial cell number > 200. (J) MICs of *E. coli* wild type, *ΔrecA*, *ΔlexA*, $lexA^{ind-}$ and *ΔsulA* strains after the ampicillin treatment for 0, 2, 4, 6 and 8 hours, respectively. All data are presented as the mean ± s.d. of six independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Mann-Whitney rank sum test.

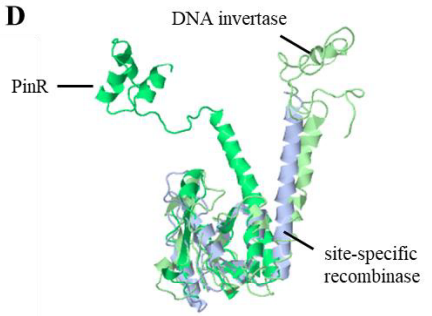


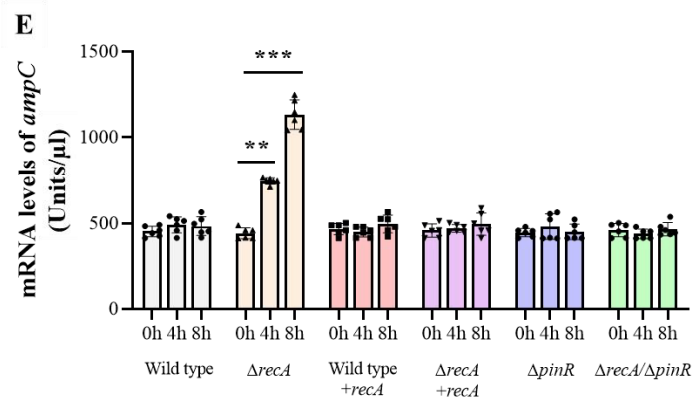
Gene mutations

Strains	Feature	Position	Mutation
Wild type			
Wild type 4h	*pinR*	1433228 1433199	T > A T > C
Wild type 8h	*pinR*	1433228 1433199 1433667	T > A T > C G > A
ΔrecA			
ΔrecA 4h	*pinR*	1433667	G > A
ΔrecA 8h	*pinR*	1433228 1433199 1433667	T > A T > C G > A









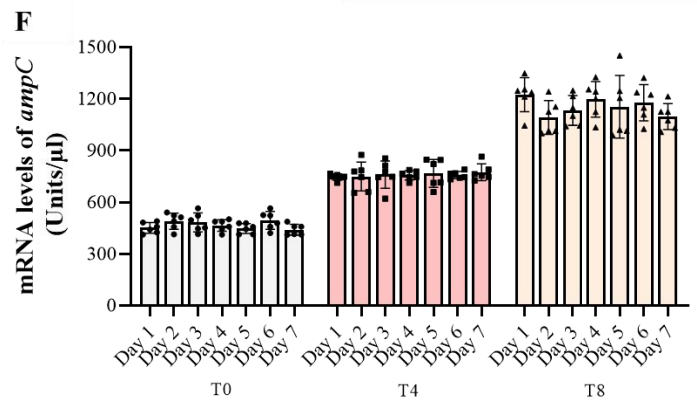


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Figure 4. *pinR* plays a decisive role in the evolution of resistance to β-lactam antibiotics. (A) Summary of whole genome sequencing result. Sequencing coverage for each bacterial isolate is plotted, according to the color: blue, non-treatment; green, 4 hours treatment; red, 8 hours treatment. Three SNPs on gene *pinR* were found in wild type and *ΔrecA* strains with initial inoculation of 1×10^9 *E. coli*. (B) MICs of *E. coli* wild type, *ΔrecA*, *ΔpinR*, and *ΔrecA/ΔpinR* strains batch cultures after a single exposure of ampicillin for 0, 4 and 8 hours, respectively. (C) MICs of *E. coli* wild type, *ΔrecA*, *ΔpinR*, and *ΔrecA/ΔpinR* strains batch cultures after a cyclic treatment of ampicillin at 50 μg/ml for 21 days. (D) Comparison of the protein structure among predicted protein PinR (phyre[2]), protein DNA invertase, and protein site-specific recombinase structure. (E) mRNA levels of gene *ampC* in all *E. coli* strains after a single exposure of ampicillin at 50 μg/ml for 0, 4 and 8 hours, respectively. (F) mRNA levels of gene *ampC* in *ΔrecA* ancestral and resistant isolates and their daughter cells. All data are presented as the mean ± s.d. of 6 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Mann-Whitney rank sum test.

Reference

1. Levy, S.B. et al. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med*. **10**, S122 (2004).

2. Alekshun, M. N. et al. Molecular mechanisms of antibacterial multidrug resistance. *Cell*. **128**, 1037-1050 (2007).

3. Ofer, F. et al. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*. **513**, 418-421 (2014).

4. Imane, E. M. et al. Stochastic expression of a multiple antibiotic resistance activator confers transient resistance in single cells. *Scientific Reports.* **6**, 19538 (2016).

5. Irit, L. et al. Antibiotic tolerance facilitates the evolution of resistance. *Science*. **355**, 826-830 (2017).

6. Levin, B. R. et al. Non-inherited antibiotic resistance. *Nat. Rev. Microbiol.* **4**, 556-562 (2006).

7. Pavlopoulou, A. RecA: a universal drug target in pathogenic bacteria. *Froun. Biosci.* **23**, 36-42 (2018).

8. Chen, Z. et al. Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *Nature*. **453**, 489-494 (2008).

9. Zgur-Bertok, D. DNA damage repair and bacterial pathogens. *PLoS Pathog*. **9**, e1003711 (2013).

10. Barrett, T. C. Enhanced antibiotic resistance development from fluoroquinolone persisters after a single exposure to antibiotic. *Nat Commun.* **10**, 1177 (2019).

11. Safo, M. K. et al. Crystal structures of the BlaI repressor from *Staphylococcus aureus* and its complex with DNA: insights into transcriptional regulation of the bla and mec operons. *J. Bacteriol.* **187**, 1833-1844 (2005).

12. Jacobs, C. et al. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell*. **88**, 823-832 (1997).

13. Jaurin, B. et al. *ampC* cephalosporinase of *Escherichia coli* K-12 has a different evolutionary origin from that of beta-lactamases of the penicillinase type. *Proc. Natl. Acad. Sci. U.S.A*. **78**, 4897-4901 (1981).

14. Jaurin, B. et al. The *E. coli* beta-lactamase attenuator mediates growth rate-dependent regulation. *Nature.* **290**, 221-225 (1981).

15. Zeng, X. et al. Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front Microbiol.* **4**, 1-9 (2013).

16. Mehta, P. et al. Analysis of the lambdoid prophage element e14 in the E. coli K-12 genome. *BMC Microbiol*. **4**, 4 (2004).

17. Kelley, L. A. et al. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* **10**, 845-858 (2015).

18. Sandegren, L. et al. Bacterial gene amplification: implications for the evolution of antibiotic resistance. *Nat. Rev. Microbiol.* **7**, 578-588 (2009).

19. Edlund, T. et al. Isolation and characterization of DNA repetitions carrying the chromosomal β-lactamase gene of *Escherichia coli* K-12. *Mol. Gen. Genet*. **173**, 115-125 (1979).

20. Edlund, T. et al. Recombination between short DNA homologies causes tandem duplication. *Nature*. **292**, 269-271 (1981).

21. Alam, M. K. et al. RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance. *Cell Chem. Biol.* **23**, 381-391 (2016).

22. Thi, T. D. et al. Effect of *recA* inactivation on mutagenesis of *Escherichia coli* exposed to sublethal concentrations of antimicrobials. *J Antimicrob Chemother*. **66**, 531-538 (2011).

23. Steward, K. F. et al. PinR mediates the generation of reversible population diversity in *Streptococcus zooepidemicus*. *Microbiology*. **161**, 1105-1112 (2015).

Supplementary information

Methods

Bacterial strains, media and antibiotics. Bacterial strains and plasmids used in this work are described in Supplementary Table 1 and Table 2. Luria-Bertani (LB) was used as broth or in agar plates. Whenever possible, antibiotic stock solutions were prepared fresh before the use.

Evolutionary protocol of the exposure to antibiotics. An overnight culture (0.6 ml; 1 x 10^9 bacterial cells) was diluted 1:50 into 30 ml LB medium supplemented with 50 μg/ml ampicillin, 1mg/ml penicillin G, or 200 μg/ml carbenicillin and incubated at 37ºC with shaking at 250 rpm. After the killing, the antibiotic-containing medium was removed by washing twice in LB medium. The survived isolates were used for analysis or stored at -80ºC for future use. For cyclic exposure (revised protocol from ref. **1**), after the washing, the culture was then resuspended in 30 ml fresh LB medium and grown overnight at 37ºC with shaking at 250 rpm. The regrown culture was repeated to be killed following by same protocol until the resistance is established.

Antibiotic susceptibility test. Each column in a 96 wells plate is filled with fresh LB medium supplemented with gradient concentration of ampicillin and inoculated with approx. 10^4 ~10^5 bacterial cells/well. The plate is incubated overnight at 37ºC with shaking (Tecan Spark, Switzerland). The Minimal Inhibitory Concentration (MIC) is the highest concentration supporting visible growth [**2**].

For MDK_{99} measurement, each column in a 96 wells plate is filled with fresh LB medium supplemented with 100 μg/ml ampicillin and inoculated with approx. 10^4 bacterial cells/well. After certain hours incubation, the plate is spun down to terminate

the antibiotic exposure by washing away antibiotic remains and resuspending in fresh medium. The plate is then returned for overnight incubation. Empty wells indicate killing of >99% of the population, and an evaluation of MDK_{99} can be read directly from the plate depending on the treatment duration [**3**].

ScanLag analysis. After each treatment of ampicillin, a sample of the culture was diluted and plated on solid LB agar medium (each plate contains less than 100 bacterial cells). The plates were placed in a ScanLag setup at 37ºC. Images were obtained automatically every 15 minutes to monitor the appearance time of colonies [**4**].

Mutation frequency test. Bacterial population mutation frequency was evaluated based on the approach of Delbrück-Luria Fluctuation test. 30 µl overnight cultures (10^4 ~10^6 amounts of E. coli cells) were plated onto solid LB agar plates supplemented with or without 50 µg/ml ampicillin. The number of colonies appearing on the plates after 48 hours incubation was used to calculate the mutation rate:

$$R = \frac{n}{N} \times P \times 100\%$$

R = Mutation frequency rate

n = Numbers of colonies appearing on the medium containing ampicillin

N = Numbers of colonies appearing on the fresh medium

P = Population surviving rate

β-Lactamase detection. The level of β-lactamase was detected using a Nitrocefin disk (Sigma-Aldrich, US). A drop of bacterial culture was placed on the surface of

Nitrocefin disk. After 30 minutes inoculation, the disk was scanned to record the colour for calculation using ImageJ software.

Construction of deletion mutants. Lambda Red recombination was used to generate various gene deletion in *E. coli* strains. In brief, *E. coli MG1655* containing insertions resistance cassette to replace the open reading frame of *recA* or *pinR* was used. Primers (used in this work are listed in Supplementary Table 3) were designed approximately 50 bp upstream and downstream to genes of interest on the. Background strain was made electro-competent and transformed with recombinase plasmid pKD46 and selected on LB agar plates containing 100 µg/ml ampicillin at 30ºC. The strain containing the plasmid was made electro-competent again using LB media containing 100 µg/ml ampicillin and 0.2% arabinose at 30ºC. Amplified DNA was transformed into recipient strain by using 50 ng of DNA and 50µL of competent cells. Cells were allowed to recover in LB media for 1 hour at 30ºC. Transformation was plated onto antibiotics selected LB agar and incubated overnight at 37ºC. The newly constructed mutant strains were cured of plasmid pKD46 through incubation of LB streak plates at 42ºC overnight.

DNA and RNA extraction. Chromosomal DNA was extracted and purified using the PureLink™ Genomic DNA mini kit (ThermoFisher Scientific). Plasmid DNA was extracted and purified using PureLink™ Quick Plasmid Miniprep kit (ThermoFisher Scientific). RNA was extracted from the cell pellets using a PureLink RNA minikit (Invitrogen). Concentration of genomic and plasmid DNA was determined on the Nanodrop™ spectrophotometer.

Droplet digital PCR (ddPCR). Genomic DNA samples were added to the Bio-Rad 2x ddPCR supermix at amounts of 0.05 ng DNA per 22 µl ddPCR reaction according to the ddPCR Bio-Rad user manual. Sample were converted into droplets using Bio-

Rad QX200 droplet generator. After the droplet generation, the plate was transferred to a thermal cycler and reactions were run under the standard cycling conditions. After the PCR, the plate was loaded onto the Bio-Rad QX200 Droplet Digital reader, and data analysis was performed using Bio-Rad QuantasoftTM software.

Copy number variations (CNVs) detection. In QuantaSoft^{TM} software, copy number is determined by calculating the ratio of the target DNA concentration to the reference DNA concentration, times the number of copies of reference species in the genome based on the below Equation. The error bars on a CN estimate in QuantaSoftTM software are the 95% confidence interval of this measurement.

$$CN = \frac{A}{\mathrm{B}} N_B$$

A = Concentration of target species

B = Concentration of reference species

N_B = Number of copies of reference loci in the genome

mRNA quantification. Reverse transcription (RT) of RNA to cDNA was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per the manufacturer's instructions. cDNA sample is then partitioned into 20,000 droplets using Bio-Rad ddPCR setup, with target and background RNA randomly distributed among the droplets. After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target RNA was present or not present after partitioning. Each droplet provides an independent digital measurement. Positive and negative droplets are counted, and software calculates the concentration of target RNA as copies/µl.

Whole genome sequencing. Genomic sequencing is explored following the Nextera Flex library preparation kit process (Illumina, USA) with adaptation described in Giao *et al*., [5]. Briefly, genomic DNA was quantitatively assessed using Quant-iT picogreen dsDNA assay kit (Invitrogen, USA). The sample was normalised to the concentration of 1 ng/µl. 10 ng of DNA was used for library preparation. After tagmentation, the tagmented DNA was amplified using the facility's custom designed i7 and i5 barcodes, with 12 cycles of PCR. The quality control for the samples was done by sequencing a pool of samples using MiSeq V2 nano kit – 300 cycles. Briefly, after library amplification, 3 µl of each library was pooled into a library pool. The pool is then clean up using SPRI beads following the Nextera Flex clean up and size selection protocol. The pool was then sequenced using MiSeq V2 nano kit (Illumina, USA). Based on the sequencing data generated, the read count for each sample was used to identify the failed libraries (i.e. libraries with less than 100 reads). Moreover, based on the read count, libraries were pooled at a different amount to ensure equal representation in the final pool. The final pool was sequenced on Illumina NovaSeq 6000 Xp S4 lane, 2x150 bp at Ramaciotti Centre for Genomics (University of New South Wales, Australia).

STORM microscopy. Bacterial chromosome was labelled using a Click-iT EdU kit following manufacturers instruction (ThermoFisher, US). DNA polymerase I was immune-labelled by primary antibody against goat and secondary antibody conjugated with Cy3B-NHS and Alexa 647-NHS as described before [6]. An imaging buffer (100 mM Tris/HCl pH 8.0, 20 mM NaCl and 10% Glucose, all purchased from Sigma-Aldrich) and an oxygen scavenger system (60 mg/ml Glucose oxidase and 6 mg/ml catalase, both purchased from Sigma-Aldrich) were sued for the STORM imaging [6]. 140 mM β-mercaptoethanol was added to promote photo-switching. The two-colour STORM imaging was sequentially acquired for up to 50,000 frames under the excitation of 647 nm and 561 nm lasers at the power density of 3 ~5 kW/cm^2 and under the photo-activation of 405 nm laser (Coherent Inc.) at the power density of 0.5

kW/cm^2 at the sample. STORM image analysis, drift correction, image rendering, protein cluster identification and images presentation were performed using Insight3 (gift of Prof. Bo Huang from UCSF), custom-written Matlab (2012a, MathWorks) codes, SR-Tesseler (IINS, Interdisciplinary Institute for Neuroscience), and Image J.

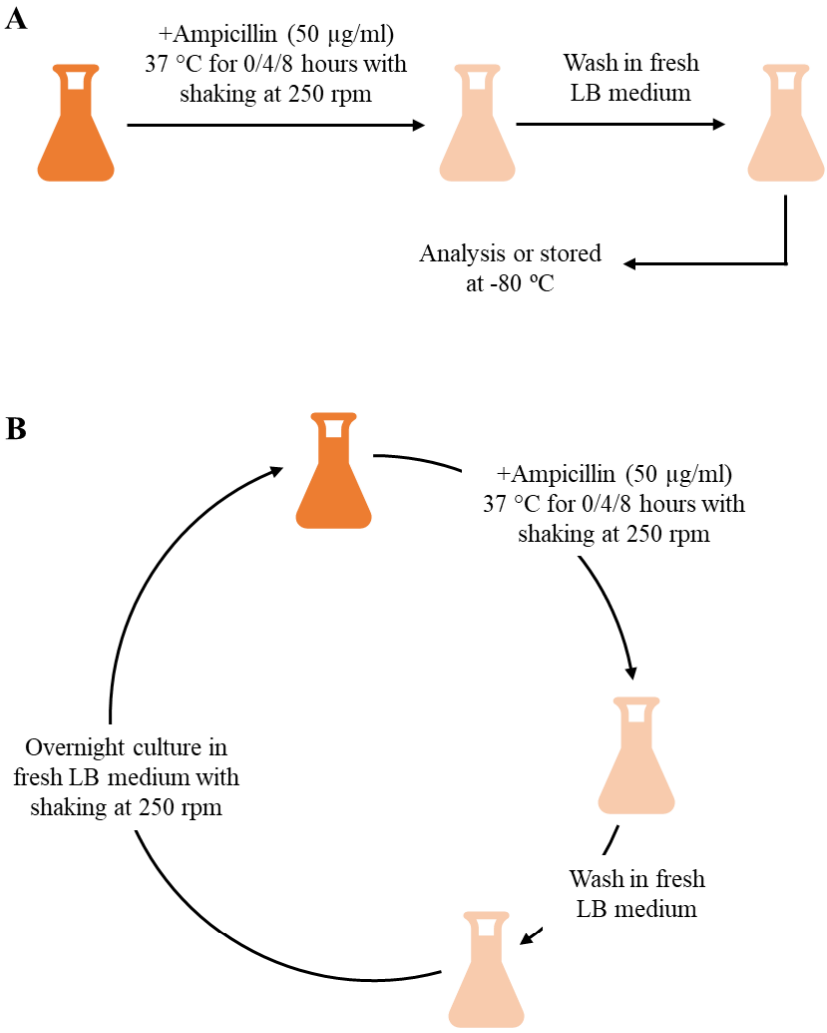

A
+Ampicillin (50 µg/ml)
37 °C for 0/4/8 hours with
shaking at 250 rpm
Wash in fresh
LB medium
Analysis or stored
at -80 ℃
B
+Ampicillin (50 µg/ml)
37 °C for 0/4/8 hours with
shaking at 250 rpm
Wash in fresh
LB medium
Overnight culture in
fresh LB medium with
shaking at 250 rpm

Figure S1. Schematic for the experimental protocol. (A) Single exposure experiment. Overnight culture is diluted into medium with lethal concentration of ampicillin (50 μg/ml). After 4 or 8 hours treatment, the culture is washed twice and analyzed or stored. (B) Cyclic exposure experiment. In each daily cycle, the overnight culture is diluted into medium with lethal concentration of ampicillin at 50 μg/ml. After 4 or 8 hours exposure, the culture is washed twice and resuspendend in fresh medium and regrown overnight to stationary phase.

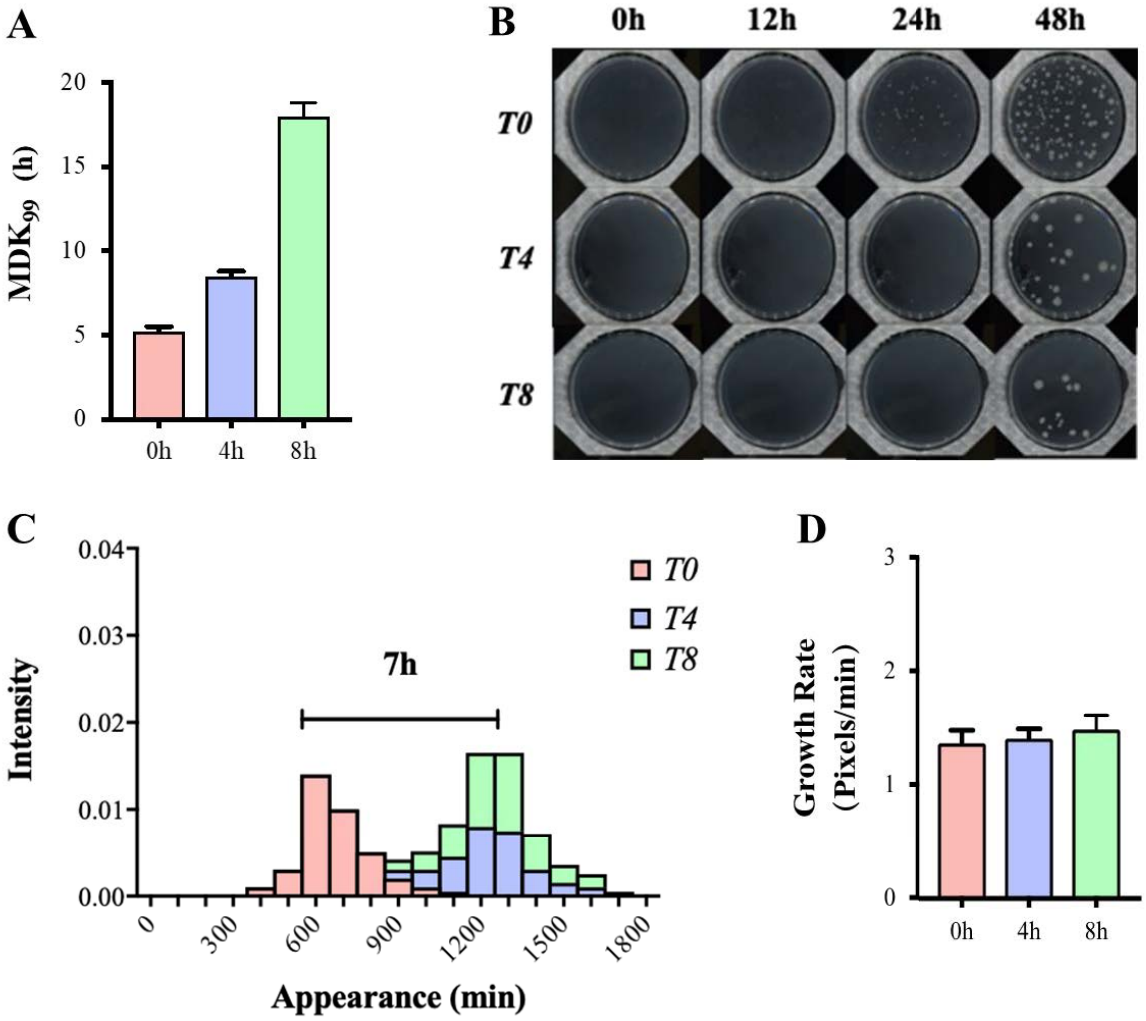

A
MDK99 (h)
20
15
10
5
0
0h
4h
8h
B
0h
12h
24h
48h
T0
T4
T8
C
Intensity
0.04
0.03
0.02
0.01
0.00
7h
T0
T4
T8
0
300
600
900
1200
1500
1800
Appearance (min)
D
Growth Rate
(Pixels/min)
3
2
1
0
0h
4h
8h

Figure S2. Emergence of tolerance in wild type strain. (A) MDK_{99} of wild type strain after the ampicillin treatment for 0, 4, and 8 hours, respectively. (B) Detection of colony appearance and growth time using automated Scanlag system on the evolved tolerant cells. (C) The histograms show the proportion of colony-forming units (CFUs) detected at each time point from 0 to 48 hours incubation. (D) Colonies grow at the same growth rate. All data are presented as the mean ± s.d. of 4 independent experiments.

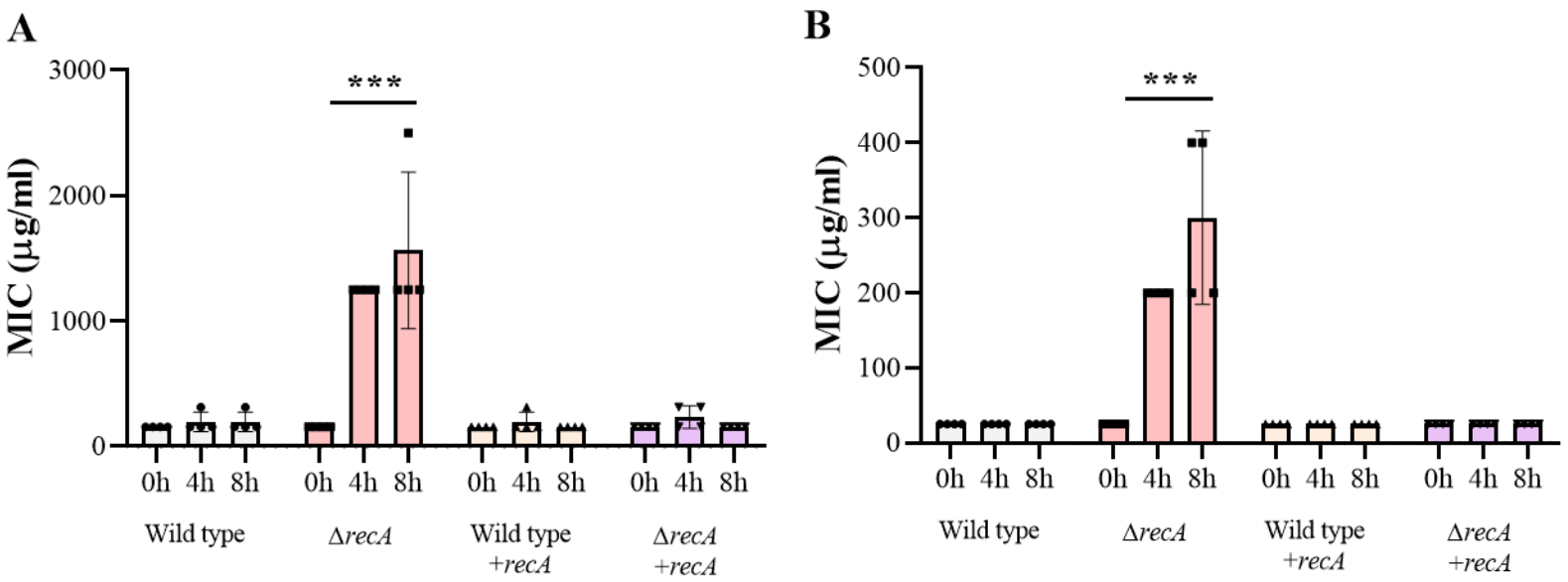


Figure S3. Emergence of resistance induced by exposure to other β-lactams antibiotics. MICs of all strains after the (A) penicillin G at 1mg/ml, and (B) carbenicillin at 200 μg/ml treatment for 0, 4, and 8 hours, respectively. All data are presented as the mean ± s.d. of 4 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Mann-Whitney rank sum test.

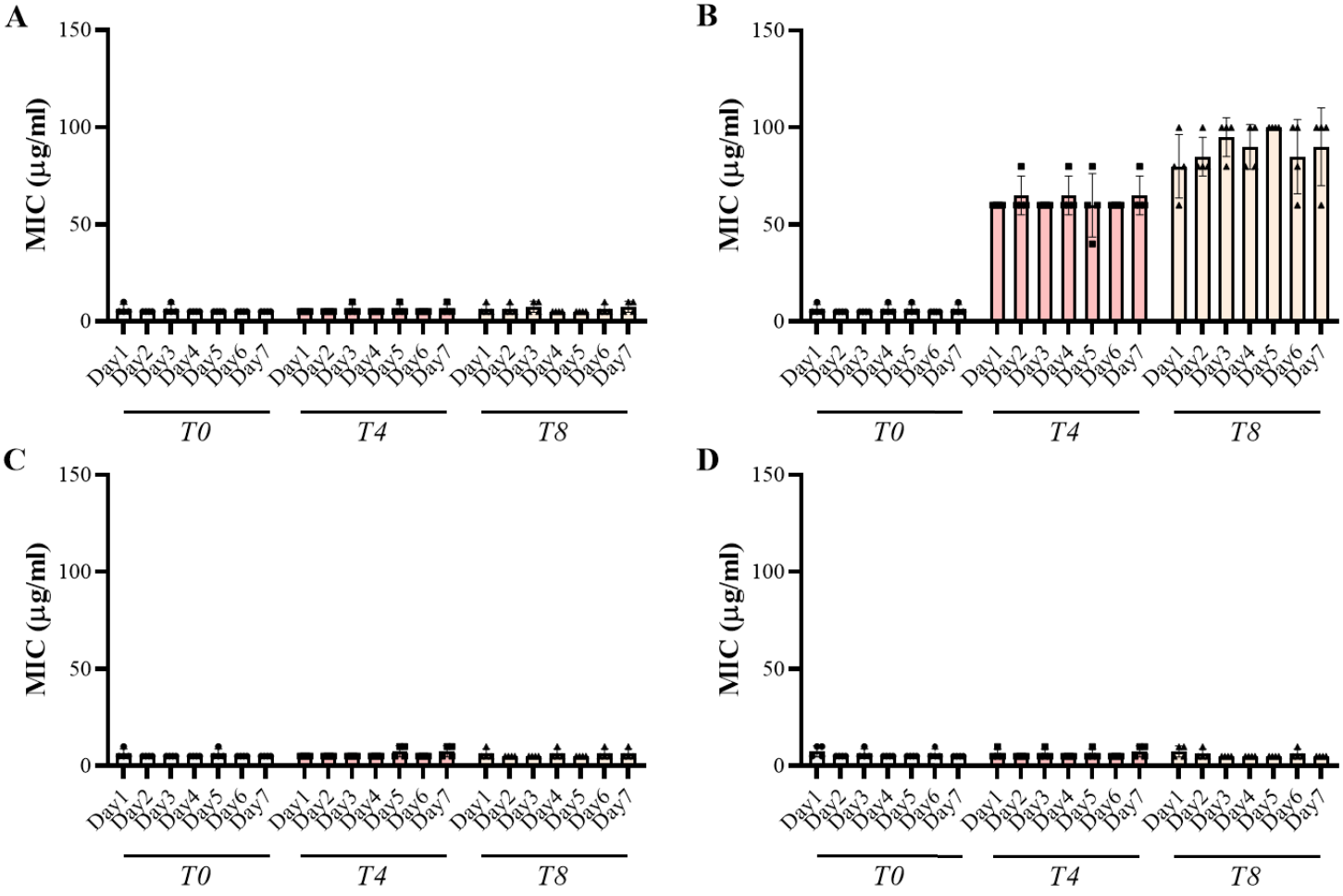

A
B
C
D
MIC (µg/ml)
150
100
50
0
Day1
Day2
Day3
Day4
Day5
Day6
Day7
T0
T4
T8

Figure S4. MIC values in daughter cells. After the exposure of ampicillin for 4 and 8 hours, respectively, the survived cells are re-suspended in fresh medium and regrown continuously for 7 days. The MIC value is measured at each day in (A) wild type, (B) *ΔrecA*, (C) *recA* overexpression, and (D) *ΔrecA* with expression of exogenous *recA*. All data are presented as the mean ± s.d. of 4 independent experiments.

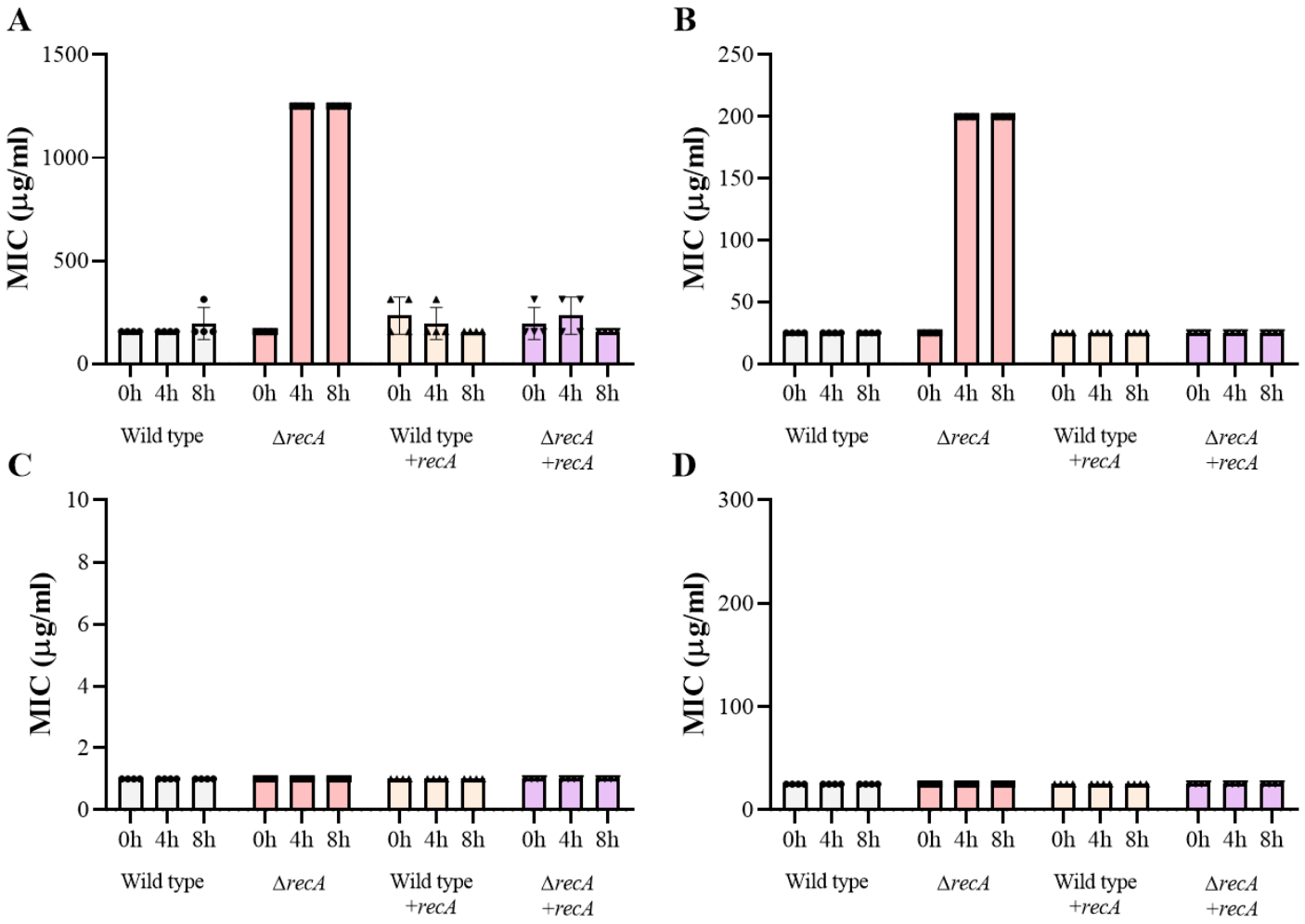

A
MIC (μg/ml)
1500
1000
500
0
0h 4h 8h 0h 4h 8h 0h 4h 8h 0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
B
MIC (μg/ml)
250
200
150
100
50
0
0h 4h 8h 0h 4h 8h 0h 4h 8h 0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
C
MIC (μg/ml)
10
8
6
4
2
0
0h 4h 8h 0h 4h 8h 0h 4h 8h 0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
D
MIC (μg/ml)
300
200
100
0
0h 4h 8h 0h 4h 8h 0h 4h 8h 0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA

Figure S5. Emergence of cross-resistance. After the exposure of ampicillin for 4 and 8 hours, respectively, the survived cells were measure for their resistance for two more β-lactams antibiotics, (A) penicillin G (MIC at 100 μg/ml) and (B) carbenicillin (MIC at 20 μg/ml), and two other classes of antibiotics, (C) ciprofloxacin (MIC at 1 ng/ml) and (D) chloramphenicol (MIC at 25 μg/ml). All data are presented as the mean ± s.d. of 4 independent experiments.

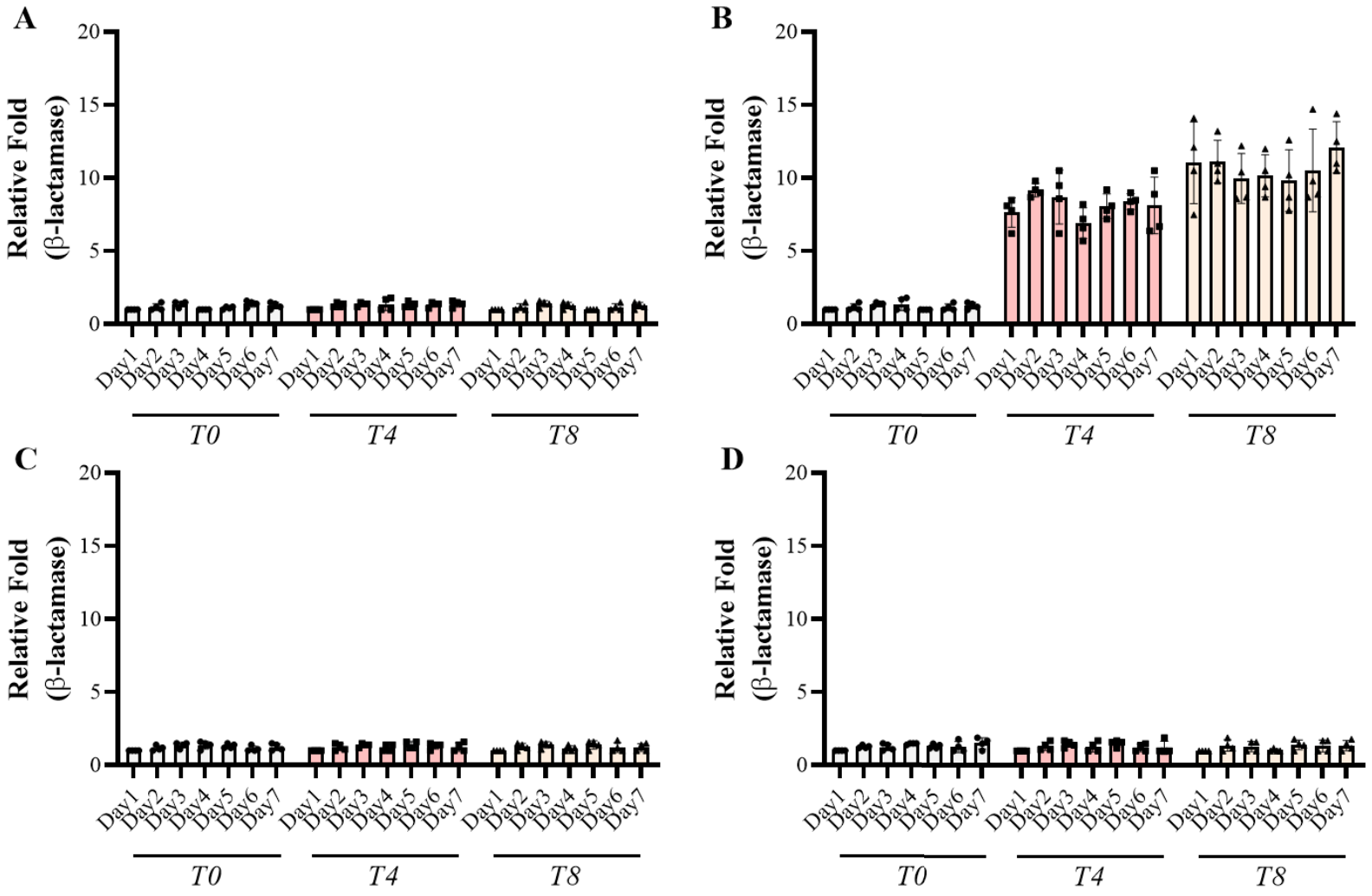

A
B
C
D
Relative Fold
(β-lactamase)
0
5
10
15
20
Day1
Day2
Day3
Day4
Day5
Day6
Day7
T0
T4
T8

Figure S6. Levels of β-lactamases in *ΔrecA* resistant daughter cells. After the exposure of ampicillin for 4 and 8 hours, respectively, the survived cells are re-suspended in fresh medium and regrown continuously for 7 days. The level of β-lactamase is measured at each day in (A) wild type, (B) *ΔrecA*, (C) *recA* overexpression, and (D) *recA* restoring strains. All data are presented as the mean ± s.d. of 4 independent experiments.

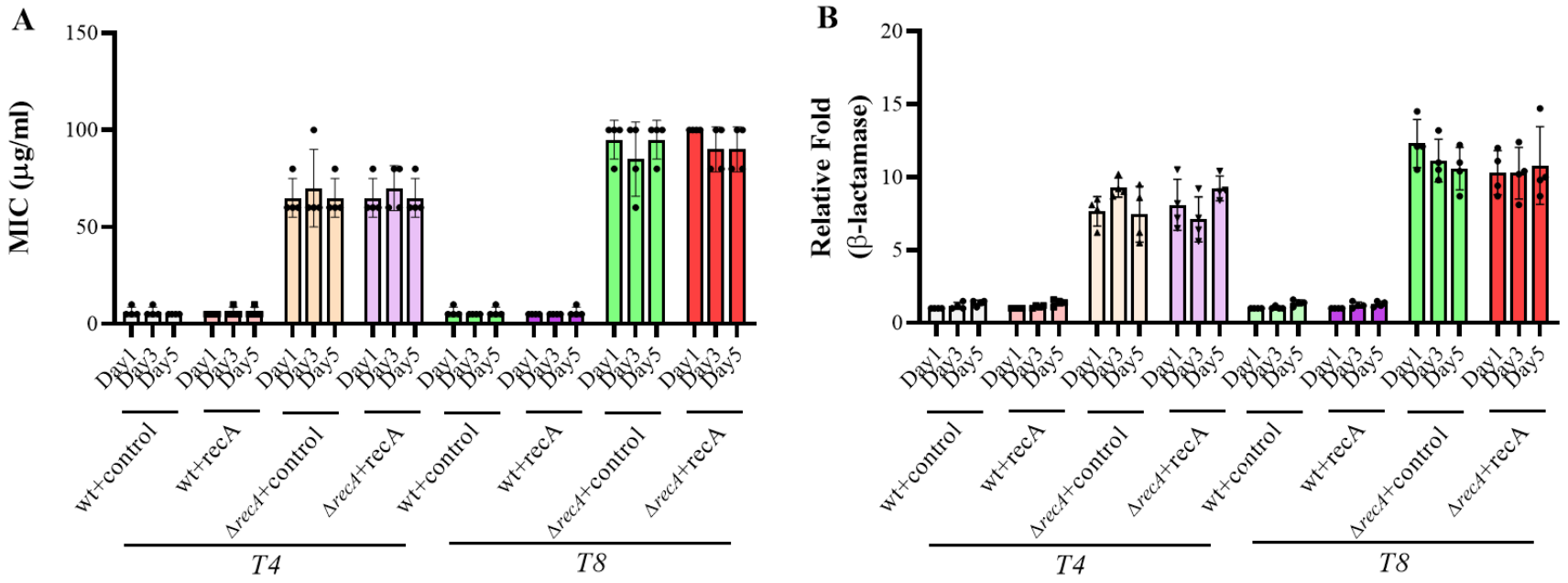


Figure S7. Establishment of resistance is prior to expression of RecA. (A) MIC value in wild type, *ΔrecA*, *recA* overexpression, and *recA* restoring daughter cells transferred with a blank plasmid (control) or plasmid with the expression of *recA* restoring. (B) Levels of β-lactamase in wild type, *ΔrecA*, *recA* overexpression, and *recA* restoring daughter cells transferred with a blank plasmid (control) or plasmid with the expression of exogenous *recA*. All data are presented as the mean ± s.d. from four individual experiments.

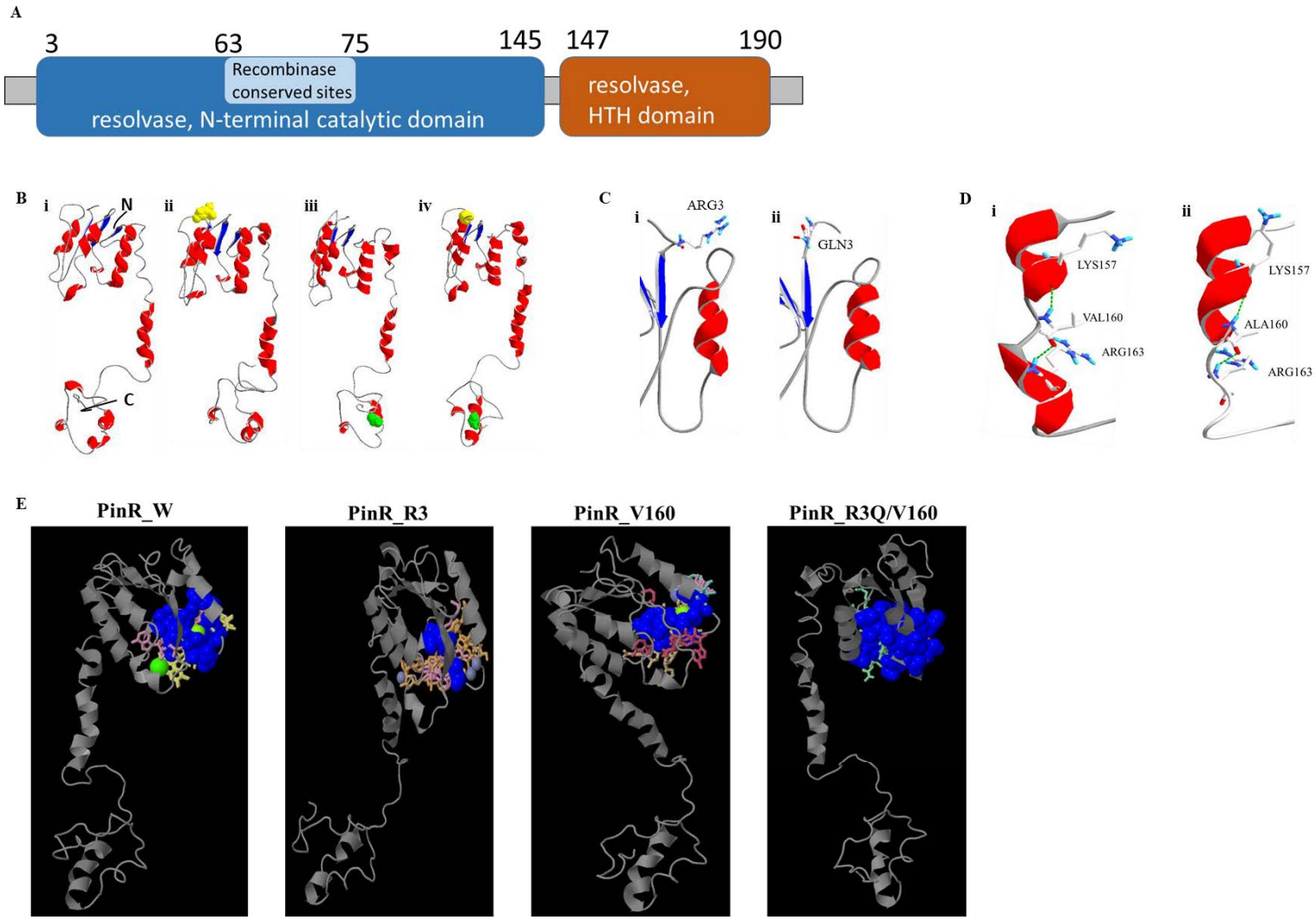

A
3
63
75
145
147
190
Recombinase conserved sites
resolvase, N-terminal catalytic domain
resolvase, HTH domain
B
i
N
C
ii
iii
iv
C
i
ARG3
ii
GLN3
D
i
LYS157
VAL160
ARG163
ii
LYS157
ALA160
ARG163
E
PinR_W
PinR_R3
PinR_V160
PinR_R3Q/V160

Figure S8. PinR domain and structure prediction. (A) Protein domain of wild-type PinR predicted by interPro platform (http://www.ebi.ac.uk/interpro/). The PinR belongs to 'resolvase' family, and it contains N-terminal catalytic domain (residues 3-145) including recombinase conserved sites (residues 63-75), and C-terminal helix-turn-helix nucleic acid-binding domain (residues 147-190). (B) Homology-based modelling of (i) PinR_WT (98% of residues modelled at >90% confidence), (ii) PinR_R3Q (97% of residues modelled at >90% confidence), (iii) PinR_V160A (98% of residues modelled at >90% confidence), and (iv) PinR_R3Q/V160A (98% of residues modelled at >90% confidence), using Phyre[2] server under intensive mode. The ribbon diagrams of the three-dimensional structure were prepared using Swiss-Pdb Viewer v4.1.0. The α-helices were shown in red and the β-sheets in blue. The mutations R3Q (yellow spheres) and V160A (green spheres) appeared to not have significant effect on the protein overall tertiary structure. (C) Local structural predictions at residue 3. (i) wild-type PinR ARG3 was located on the extremity of N-terminal catalytic domain, and (ii) the mutation to GLN3 was not predicted to cause local structural changes. The mutation of a polar positively charged ARG to polar, non-charged GLN in the surface region might modify the charge of the local environment to affect the catalytic activity of PinR. (D) Local structural predictions at residue 160. (i) The wild-type PinR VAL160 was located on a short turn connected two alpha-helices that formed HTH structure for nucleic acid-binding, and formed two hydrogen bonds (green dotted lines) with LYS157 (distance 2.74 Å) and ARG163 (distance 2.70 Å); (ii) The mutation ALA160 was predicted to perturb the local secondary structure of HTH nucleic acid-binding motif by decreasing one alpha-helix structure. The mutation of VAL160 to ALA160 was predicted to form stronger hydrogen bonds with LYS157 (distance 2.44 Å) and ARG163 (distance 2.52 Å), which may increase hydrogen bond network and thereby possibly stabilize the local alpha-helix region. The mutation V160A likely affected nucleic acid-binding capacity, and therefore may influence the gene transcription mediated by PinR. (E) 3DLigandSite Models [**5**] for the predicted active sites of PinR_WT, PinR_R3Q,

PinR_V160A, and PinR_R3Q/V160A. The proteins shown in grey with potential metallic heterogens shown as space fill and non-metallic heterogens as wireframe. Residues involved in bindings are blue. Compared to wild-type PinR, the PinR_R3Q, PinR_V160A, and PinR_R3Q/V160A all showed the changes on the predicted residues responsible for forming active sites that were in N-terminal catalytic domain (see also Table S4). The mutation (s) of PinR therefore likely affected the protein's active site, thereby possibly modifying the activity of PinR.

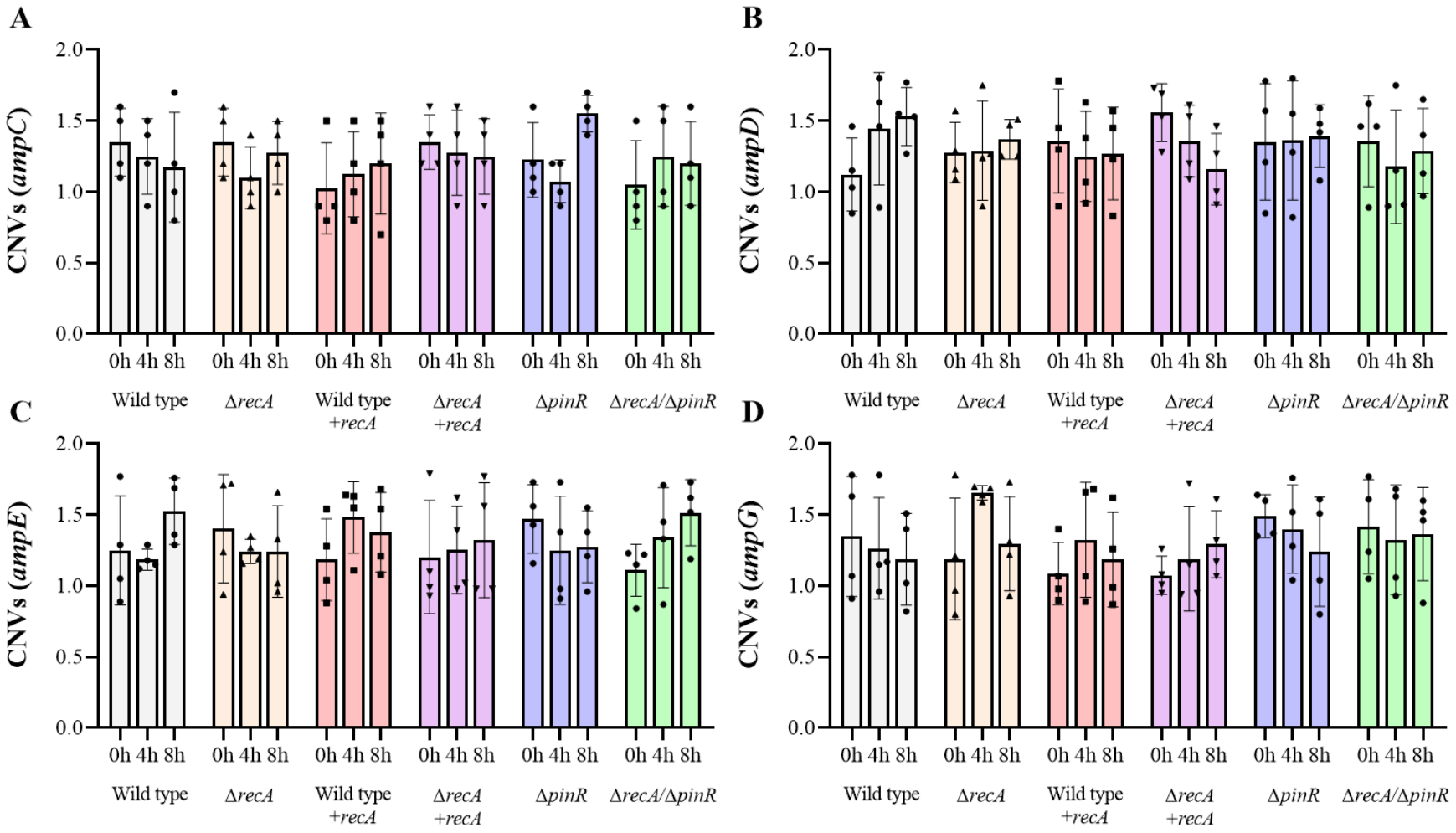
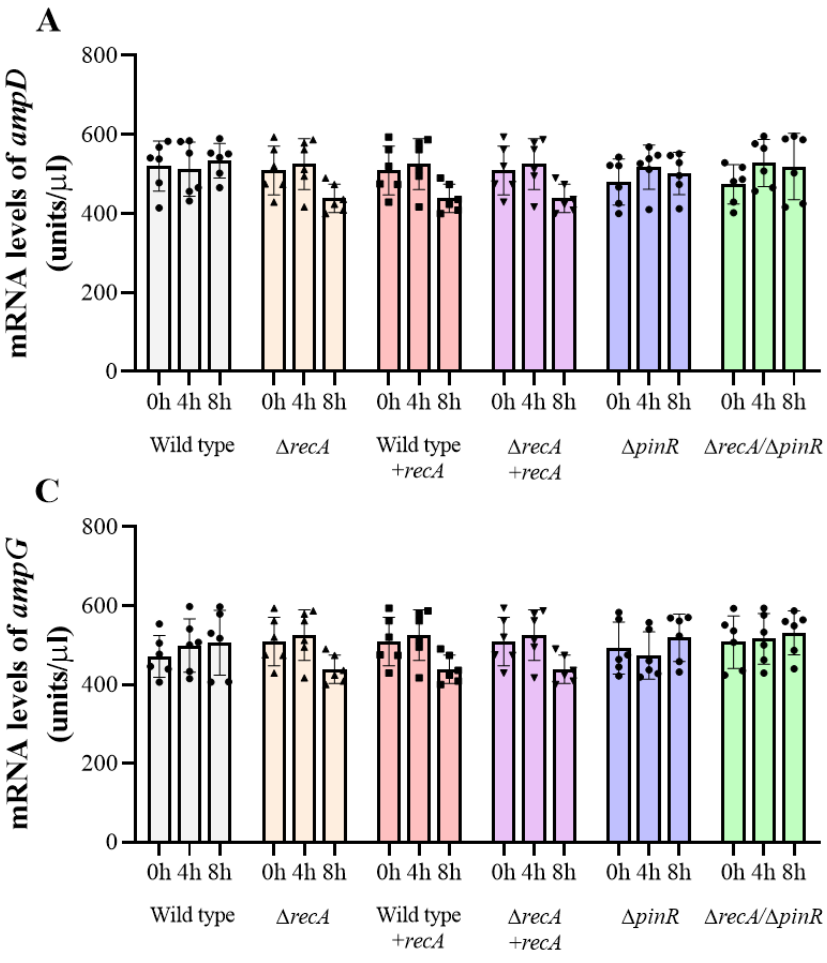
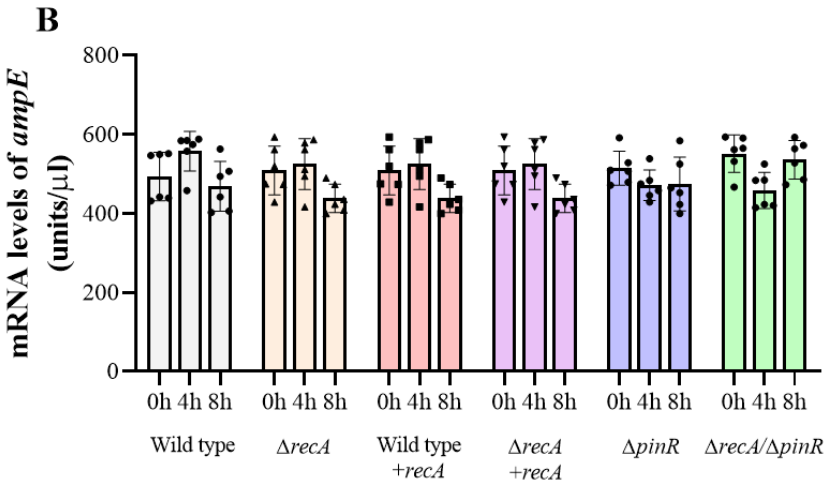

A
B
C
D
CNVs (ampC)
CNVs (ampD)
CNVs (ampE)
CNVs (ampG)
2.0
1.5
1.0
0.5
0.0
0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
ΔpinR
ΔrecA/ΔpinR

Figure S9. DNA copy number variations. DNA copy number variations of (A) *ampC*, (B) *apmD*, (C) *ampE*, and (D) *ampG* in all *E. coli* strains after a single exposure of ampicillin at 50 μg/ml for 0, 4 and 8 hours, respectively. All data are presented as the mean ± s.d. from four individual experiments.


A
mRNA levels of ampD (units/μl)
800
600
400
200
0
0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
ΔpinR
ΔrecA/ΔpinR


B
mRNA levels of ampE (units/μl)
800
600
400
200
0
0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
ΔpinR
ΔrecA/ΔpinR

C
mRNA levels of ampG (units/μl)
800
600
400
200
0
0h 4h 8h
Wild type
ΔrecA
Wild type +recA
ΔrecA +recA
ΔpinR
ΔrecA/ΔpinR

Figure S10. Bacterial mRNA levels. mRNA levels of (A) *ampD*, (B) *apmE*, and (C) *ampG* in all *E. coli* strains after a single exposure of ampicillin at 50 μg/ml for 0, 4 and 8 hours, respectively. All data are presented as the mean ± s.d. from four individual experiments.

Supplementary Table 1. Strains

Strain	***Genotype and Relevant Characteristics***	***Source***
DH5α	F-*hsdR17 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1*	Lab stock
MG1655	Rph1 ilvG rfb-50	Lab stock
BW25113 (*wild-type*)	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Lab stock
ΔrecA	MG1655*ΔrecA*	This study
ΔpinR	MG1655*ΔpinR*	This study
ΔrecA/ΔpinR	MG1655*ΔrecA/ΔpinR*	This study
JW0941-1 (*ΔsulA*)	BW25113*ΔsulA*	Keio collection
EAW26 (*ΔlexA*)	MG1655*ΔlexA*	Lab stock
RW1570 (*lexA^{ind-}*)	MG1655*lexA^{ind-}*	Lab stock

Supplementary Table 2. Plasmids

Plasmid	***Description***	***Source***
pJM1071-*recA*-clone	Gene *recA* cloned into the MCS of pJM1071, spectinomycin resistance	This study
pKD3-*pinR*-clone	Gene *pinR* cloned into the MCS of pKD3, chloramphenicol resistance	This study
pBR322-*recA*-clone	Gene *recA* cloned into the MCS of pBR322, tetracycline resistance	This study
pKD4-*pinR -clone*	Genes *pinR, pinQ* cloned into the MCS of pKD4, kanamycin resistance	This study

Supplementary Table 3. Primers

Gene	*Forward or Reverse*	*Sequence (5`-3`)*	*Annealing Temp (ºC)*
recA	F	AAAAAAGCAAAAGGGCCGCAGATGCGACCCTTGTGTATCAAACAAGACGAGAAACG AGAGAGGATGCTCAC	55
	R	CAACAGAACATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAAGACGTCTA AGAAACCATTATTATCATGAC	58
pinR	F	CAAGGAAGGGGGCTTGGAAGACGTAAAGCATCTCACACCGAGATTATTTTCATATGAA TATCCTCCTTAGTTCCTATTC	57
	R	TTTCTGAGATGCATTATGATATGAACACCAATTTCGTATAGAGTCTCACTGAGCTGCTTC GAAGTTCCTA	55
ampC	F	TTA CTT TAC CTG GGG CTA TG	59.9
	R	GTG GGA TCG CTT AAC TTG A	60.3
ampD	F	GAT CGA CGC ATT ATT CAC TG	60.9
	R	CGA AAG GAA CAT ACT GGA CTA	59.6
ampE	F	GGC GTG ACT TTT TTA CTG TT	59.6
	R	ACT TTA CCT GCG CCA ATA C	60.2
ampG	F	CAT TGG TTT CTT CTC TCT GGT	60.7
	R	AAT GGC GAC TAA TAA CAG GA	59.9
cysG	F	CGA AAA ACT TGA ATC ACT GC	60.6

R	AAT GGC TTT CTG ATC GTT G	60.3

Supplementary Table 4. 3DLigandsite active site predictions

Protein	*Predicted binding site*	*Heterogens*
PinR_wt	AGR9, ILE10, SER11, THR12, GLN15, THR16, ASN19, GLN20, LYS70, ARG73	COA, GAL, ADP, FMN, MG, CA
PinR_R3Q	SER11, ASP72, ARG73	FMN, PLP, ZN, GAL, COA, ADP, SAM, FRU, NAD
PinR_V160A	ARG9, ILE10, SER11, GLN15, THR16, ASP72, ARG73	MG, ZN, GAL, COA, SAM, ADP, ARA, ADX, NAD
PinR_R3Q/V160A	ARG9, ILE10, SER11, THR12, GLU39, HIS40, ILE41, SER42, ALA46, THR47, SER48, ARG50, PRO51, ASP72, ARG73, LEU74, GLY75, CYS76, ASN77	COA, GAL, NDP, MG, FMN, ADP, NAD

Reference

1. Irit, L. et al. Antibiotic tolerance facilitates the evolution of resistance. *Science*. **355**, 826-830 (2017).
2. Scholar, E. M. et al. The Antimicrobial Drugs. *Oxford Univ. Press*. (2000).
3. Nathalie, Q. B. et al. Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol*. **17**, 441-448 (2019).
4. Levin-Reisman, I. et al. ScanLag: high-throughput quantification of colony growth and lag time. *J. Vis. Exp*. **89**, (2014).
5. Wass, M. N. et al. 3DLigandSite: predicting ligand-binding sites using similar structures. *Nucleic. Acids. Res.* **38**, 469-473 (2010).