Microbiological Research 295 (2025) 128106

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Starvation-induced mutagenesis in *rhsC* and *ybfD* genes extends bacterial tolerance to various stresses by boosting efflux function

Yingkun Wan [a], Lianwei Ye [a,c], Jiaqi Zheng [a], Yang Tang [a,c], Edward Wai-Chi Chan [a], Sheng Chen [a,b,*]

[a] State Key Lab of Chemical Biology and Drug Discovery and the Department of Food Science and Nutrition, The Hong Kong Polytechnic University, Kowloon, Hong Kong
[b] Shenzhen Key Lab of Food Microbial Safety Control, The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, China
[c] Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong

ARTICLE INFO

Keywords:
Antibiotic tolerance
Tolerant subpopulations
Genetic changes
Mutagenesis
Efflux

ABSTRACT

Recent evidence showed that bacteria actively maintained a range of physiological functions to enhance survival fitness under adverse growth conditions. In this study, we investigated whether bacteria need to undergo active genetic changes for stress-protection purposes if environmental stress persists. Our results revealed that mutations became detectable at specific sites in several genes in *E. coli* after encountering starvation conditions for six days. This discovery is groundbreaking since bacteria are not known to undergo site-specific mutagenesis during prolonged starvation when most physiological activities are down-regulated. The genes in which mutations were consistently detected in the tolerant population were *ybfD* and *rhsC* within the *ybf* gene cluster, which are predicted to encode components of a transporter. To assess the impact of these mutations on bacterial survival, mutants with single or double mutations in these genes were generated and tested. The results demonstrated that these mutations caused significant increase in tolerance to antibiotics, heat, and oxidative stresses. Functional analysis indicated that the *E. coli* BW25113::*ybfDrhsC* double mutant exhibited elevated efflux activity, and that expression of the *rhsC* gene was suppressed in the *E. coli* BW25113:: *ybfD* mutant, suggesting that mutations in these two genes act synergistically to strengthen the stress tolerance phenotype. Consistently, deletion of the *ybfD* and *rhsC* genes resulted in significantly reduced tolerance under prolonged starvation conditions. Understanding the mechanisms of bacterial site-specific mutagenesis that enable bacteria to withstand multiple stresses over extended periods could aid development of innovative antimicrobial strategies.

1. Introduction

Antibiotic resistance has emerged as a significant public health concern in recent decades. The emergence of multidrug-resistant bacterial strains, often referred to as superbugs, exacerbates this issue and poses a severe health threat to humans. One primary mechanism behind bacterial antibiotic resistance involves mutational changes in the genetic makeup, which lead to structural alterations in drug targets that evade the bactericidal effects of antimicrobial drugs. Currently, known antibiotic resistance-associated mutations primarily confer phenotypic resistance to antibiotics, with minimal impact on resistance to other stresses. In fact, these mutations often come at a fitness cost (Dunai et al., 2019). Mutations that result in resistance to multiple stresses could potentially render pathogenic bacteria more invasive within the host's body, yet the effects of such mutations remain poorly characterized. Whether bacteria possess molecular mechanisms that can introduce antibiotic or stress resistance-conferring mutations at specific sites when faced with prolonged antimicrobial stress is an intriguing scientific question.

Bacterial mutations are typically believed to occur randomly and spontaneously during DNA synthesis. As organisms carrying mutations that provide a survival advantage in specific environmental conditions can outgrow organisms without such mutations, this phenomenon gives a false impression that bacteria can undergo site-specific mutational changes in response to environmental stresses. Previous studies have attempted to demonstrate that bacteria can actively undergo mutagenesis in specific genes to survive particular stresses (Bjedov et al., 2003). However, none of these experiments can conclusively show that bacteria have the ability to control where in the genome mutations occur. These experiments have at best shown that bacteria can increase the rate of

* Correspondence to: The Hong Kong Polytechnic University, Hong Kong.
E-mail address: sheng.chen@polyu.edu.hk (S. Chen).

https://doi.org/10.1016/j.micres.2025.128106
Received 17 October 2024; Received in revised form 29 January 2025; Accepted 16 February 2025
Available online 21 February 2025

mutations by utilizing error-prone polymerases under stress, thereby raising the chances of developing stress-resistant genetic variants (Gressel and Levy, 2010). Other mutational mechanisms involve producing the most fitting proteins to enhance survival fitness through mRNA reverse-transcription (Cairns et al., 1988) and activating genetic transposition functions (Schmitz and Querques, 2024).

The first observation of bacterial mutation was made by Luria and Delbruck in 1943 (Luria and Delbrück, 1943). In their seminal study, bacterial populations were observed to drastically decrease when attacked by viruses, resulting in a clear culture after a few hours. However, with extended incubation, a small population of bacteria consistently survived. Luria hypothesized that this population arose from a mutant with higher survival fitness. Subsequent investigations discovered that various environmental stresses accelerate the rate of genetic changes in bacteria, yielding genetic variants better equipped to survive adverse conditions. For instance, mutations commonly observed in stationary phase cultures were linked to a notable increase in DNA replication errors. This process involves the utilization of error-prone polymerase DinB and the suppression of recombinational double DNA strand break repair. Unrelated chromosomal gene mutations were also noted during this phase, indicating that stationary conditions induce mutational changes that may or may not enhance survival fitness (Bull et al., 2001).

Oxidative stress has been reported to induce gene mutagenesis in bacteria, primarily due to reactive oxygen species (ROS) generation. The response to oxidative stress, particularly H_2O_2-induced stress, involves the transcription factor OxyR and is known to cause DNA damage and mutational changes. Alkylation stress can similarly lead to genetic changes (Lagage et al., 2022). The onset of SOS-dependent spontaneous mutagenesis necessitates oxygen and entails the expression of genes *recA* and *umuC* (Bhamre et al., 2001). The work by Lenski and Mittler highlighted the occurrence of genetic mutations during starvation stress, with mutation rates gradually increasing to 10^-5 in single colonies over a 6-day starvation period during which sugar was not available (Lenski and Mittler, 1993). Adaptive mutations induced by the fusion of *ara-B-lacZ* coding-sequences were also observed during glucose starvation (Maenhaut-Michel et al., 1997). However, the specificity of stress resistance associated with such mutations remains unclear.

It is important to note that mutational changes are not the sole molecular mechanism bacteria employ to combat environmental stresses and enhance survival fitness. Bacteria actively suppress physiological activities and produce various protective enzymes and proteins when faced with adverse environmental conditions to counteract the negative effects of stressors. In 1944, fifteen years after the discovery of the first antibiotic penicillin and around the time Luria and Delbrück observed the first bacterial mutation, Joseph Bigger noted that bacteria consistently harbored a sub-population that could not be eradicated by antibiotics (Bigger, 1944), leading to creation of the term 'antibiotic tolerance'. Recent studies conducted in our laboratory demonstrated the existence of various active defense mechanisms that confer phenotypic tolerance to antibiotics and other stresses temporarily; such mechanisms involve the production of proteins that help maintain the transmembrane proton motive force, mediate active efflux to expel toxic metabolites, and those which play a role in DNA damage repair (Wang et al., 2021). These tolerance mechanisms are mainly activated upon detecting changes in the environment and do not require genetic alterations. However, if adverse conditions persist, bacterial persisters may need to undergo mutational changes to further prevent cellular damage caused by prolonged stress. The adverse environmental conditions that induce tolerance formation are likely to trigger mutational changes in the bacterial genome. Mutations in genes like *hipA* have previously been associated with tolerance formation in bacteria (Sulaiman and Lam, 2021). Yet, it remains unclear whether these mutations arise due to specific stress inductions, and the tolerance phenotypes of strains with such mutations have not been extensively characterized.

This study aims to investigate whether site-specific genetic changes occur in persisters under prolonged starvation to complement physiological tolerance mechanisms and enhance survival fitness. Prolonged starvation was chosen as a test condition because bacteria do not replicate and maintain minimal physiological activities, including nucleic acid synthesis, under such conditions. It is unlikely that new mutant strains can be generated for specific stress-resistant strain selection during starvation. Although nutrient starvation is considered a factor that induces mutations, this condition does not exert selection pressure on mutations within the test population as cellular replication does not occur. Therefore, any mutations that arise in the bacterial population under prolonged starvation stress are likely due to unique and previously unknown site-specific mutagenesis mechanisms activated by the organisms under stress. Results of this study consistently demonstrate that prolonged starvation can indeed induce mutational changes at specific sites in efflux pump-encoding genes. These findings, which suggest that bacteria possess sophisticated molecular mechanisms to adjust their genetic makeup based on the nature, severity, and persistence of environmental stress, have significant implications in development of new strategies to suppress the survival fitness and virulence of bacterial pathogens.

2. Materials and methods

2.1. Assessment of mutation induction potential of starvation stress

To investigate whether genetic changes occur in bacteria when exposed to nutrient starvation, overnight cultures of *E. coli* BW25113 (Yang et al., 2022) and W3110 (Tabata et al., 1989) were deprived of all nutrients and then subjected to whole-genome sequencing, as illustrated in Fig. 1. Initially, 200 μl of overnight culture of the test strains were added to 20 ml of LB broth and incubated at 37°C until the culture's OD600 reached 0.2. Bacteria were washed and suspended in 0.85 % saline to induce a starvation condition, following a method described in a previously reported tolerance assay (Wan et al., 2021). Bacterial samples were collected at three time points: 0 hours, 72 hours of starvation, and 144 hours of starvation. Subsequently, DNA was extracted and subjected to whole-genome sequencing.

2.2. Creation of gene knockout strains

The functions of genes in which mutations occurred during starvation were determined by creating and testing the corresponding gene knockout mutants. The plasmid pKD3, carrying the selectable antibiotic chloramphenicol resistance gene flanked by FRT (FLP recognition target) sites, was used in a double gene knockout experiment, where the target gene was replaced by the chloramphenicol resistance gene. To achieve this, competent cells of the wild-type strain were prepared by incubation at 37°C until the OD600 reached 0.3–0.5, followed by washing three times with 10 % glycerol at 4°C. The plasmid pKD46, a temperature-sensitive plasmid expressing the λ Red recombinase proteins, was then transformed into the competent cells. Ampicillin-resistant transformants were selected on agar plates containing 100 μg/ml ampicillin. Subsequently, competent cells of strains carrying pKD46 were prepared by incubating the strains at 30°C until the OD600 reached 0.3–0.4, followed by the addition of 0.5 % arabinose, incubation for 1 hour, and triple washing in glycerol at 4°C. Finally, the homologous sequence-FRT-flanked chloramphenicol resistance gene was transformed into the competent cells, and the gene knockout strains were selected on agar plates containing 50 μg/ml chloramphenicol.

2.3. Introduction of mutations into wild-type strains

In this study, mutation sites in the test strains were frequently found near the ends of the genes. To investigate the effects of these mutations, the Flp-Frt recombination approach was employed to create specific mutants. The plasmid pKD3, carrying the selectable antibiotic

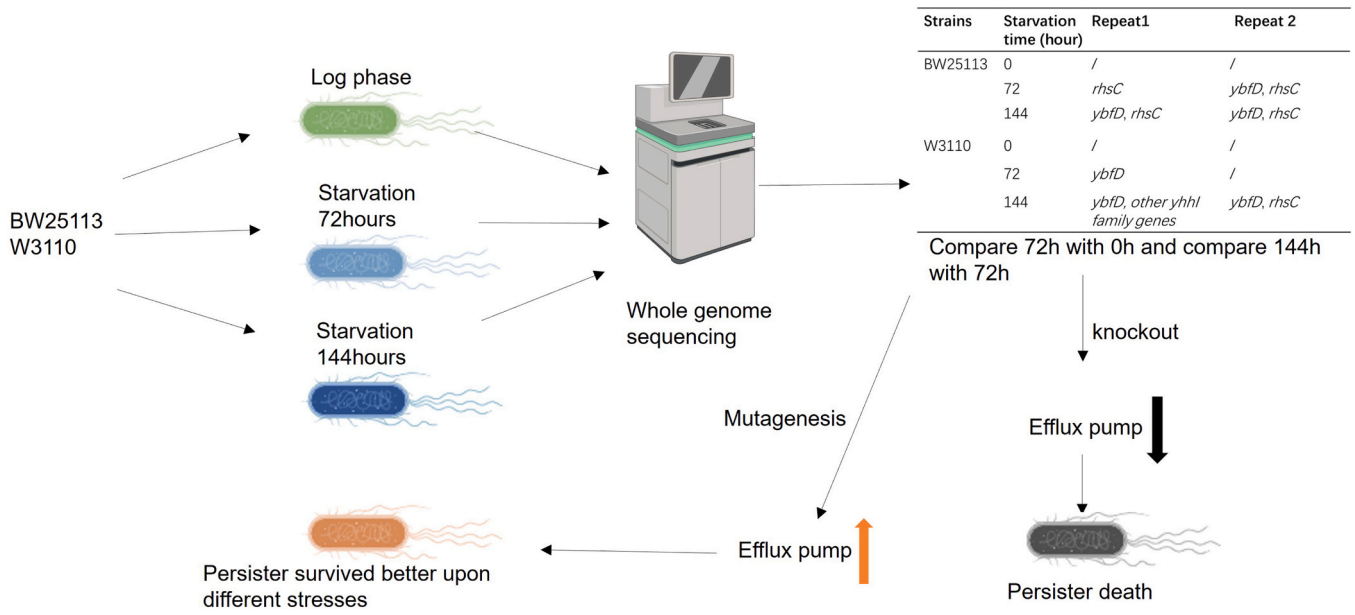

Fig. 1. Procedure of preparation of bacterial persisters for whole genome sequencing and subsequent genetic and functional analysis. Bacterial populations of strain BW25113 and W3110 were subjected to starvation for up to 144 hours; DNA were extracted from the test populations and subjected to whole genome sequencing, genomic sequences of samples collected at 72 hr were compared to those collected at 0 hr and 144 hr to identify genetic changes that occurred at different time points. Two genes in which mutational changes occurred (*ybfD* and *rhsC*) were deleted and tested for the effect on antibiotic tolerance. Deletion of these two genes was found to result in reduced efflux functions and significantly lower level of tolerance to antibiotics. Mutations identified in the *ybfD* and *rhsC* genes were also introduced into wild type strains to test their effects on antibiotic tolerance. The results showed that these mutations could confer a higher level of antibiotic tolerance.

chloramphenicol resistance gene flanked by FRT sites, was utilized in a double gene knockout experiment where the target gene was replaced by the *cat* gene. Initially, mutated DNA fragments were created, and the *cat* gene was inserted between the target gene and a 50 bp DNA fragment downstream of the target gene. Primers were designed to overlap both fragments by 20 bp to facilitate their connection. Mutated fragments and *cat* gene-bearing fragments were amplified by PCR, purified by gel extraction, and mixed in a 1:1 ratio. A fusion protocol was adopted where DNA templates were amplified by PCR without primers in PCR master mix. The templates were subjected to a gradual cooling process from 95°C to 72°C over 5 minutes to facilitate fusion of the two fragments. Subsequently, the forward primer of the mutated fragment linking the resistance-encoding sequence and the reverse primer of the 50 bp fragment were added, and 30 cycles of PCR was performed.

Next, competent cells of a single gene knockout strain were prepared by incubation at 37°C until the OD600 reached 0.3–0.5, followed by triple washing with 10 % glycerol at 4°C. The plasmid pKD46, a temperature-sensitive plasmid expressing the λ Red recombinase proteins, was transformed into the competent cells, and ampicillin-resistant transformants were selected on agar plates containing 100 μg/ml ampicillin. Subsequently, competent cells of strains carrying pKD46 were prepared by incubating the strains at 30°C until the OD600 reached 0.3–0.4, followed by the addition of 0.5 % arabinose, 1-hour incubation, and triple washing in glycerol at 4°C. Finally, the homologous sequence-FRT-flanked cat gene was transformed into the competent cells, and the double gene knockout strains were selected on agar plates containing 50 μg/ml chloramphenicol.

2.4. Quantitative PCR validation assay

To determine whether the expression level of the *rhsC* gene was regulated by the *ybfD* gene product, bacterial populations of the wild type *E. coli* BW25113, *E. coli* BW25113::*ΔybfD* knockout strain, and the *E. coli* BW25113::*ΔybfD* mutant were subjected to starvation for six days. Following starvation, RNA extraction (Invitrogen RNA extraction kit), purification (TURBO DNA-free™ kit), and conversion into cDNA (SuperScript first-strand cdna synthesis kit) via reverse transcription were performed. The cDNA of the three samples was then subjected to qPCR (PowerUp™ SYBR™ Green Master Mix) to assess the expression level of the *rhsC* gene in each strain by ABI QuantStudio 7 Flex Real-time PCR System and analysis by QuantStudio™ Design & Analysis Software.

2.5. Starvation-induced tolerance assay

The bacterial population was subjected to starvation stress using the following approach. The bacterial culture at mid-log phase was subjected to centrifugation (6000 g, 5 minutes), followed by the removal of the supernatant and resuspension of the pellet in 0.85 % saline. The cell suspension was subjected to another round of centrifugation, removal of the supernatant and resuspension in 0.85 % saline to ensure that all nutrients were removed from the cell suspension. Ampicillin (100 μg/ml) was added to the cell suspension and then every second day for up to one month. Gentamicin (10 μg/ml) was added to the cell suspension daily for up to 6 days, followed by incubation at 37°C and shaking at 250 rpm. The survival rate of the bacteria in the cell suspension was recorded by measuring the CFU (colony-forming unit) for a period of 31 days. Each assay was repeated three times, and the error bars were included in the graphs.

2.6. Membrane potential assay for gene knockout strains and mutants

An overnight culture was prepared, and 50 μl of the overnight culture was added to 5 ml LB broth and incubated at 37°C until the O.D. reached 0.2. The bacteria were washed and resuspended in 0.85 % saline to induce starvation stress. The test population was then incubated without shaking for up to 144 hours. Bacteria in the log phase were included as a control. DiSC3(5) was used as a membrane potential-sensitive probe, KCl was used as a source of protons, and valinomycin, which can disrupt the PMF, was used as a positive control. KCl and DiSC3(5) were added until a final concentration of 100 mM and 1 μM,

respectively, was reached; this step was followed by incubation at room temperature for 15 minutes in the dark to allow the dye to penetrate the outer membrane and produce a quenching effect. Valinomycin (1 μM) was then added to the positive control group. The fluorescence reading was monitored using a Clariostar Microplate Reader (BMG LABTECH) at an excitation wavelength of 622 ± 10 nm and an emission wavelength of 670 ± 10 nm for 10 minutes. Upon depolarization, the dye was rapidly released into the medium, causing dequenching and facilitating fluorometric detection.

2.7. Measurement of ATP level in knockout strains and mutants

An overnight bacterial culture was prepared, and 50 μl of the overnight culture was added to 5 ml LB broth and incubated at 37°C with shaking until a cell density of OD value 0.2 was reached. The diluted culture was then incubated at 37°C, followed by washing and resuspending the cell pellet in 0.85 % saline. The bacterial suspension was incubated for 144 hours, during which the test population was subjected to starvation stress. Bacteria in the log phase were included as a control. Bacteria in these four groups were lysed to determine the ATP level. In this experiment, 1 ml of 1 × Reaction Buffer was prepared by adding 50 ml of 20 × Reaction Buffer (Component E) to 950 μl of deionized water. 1 ml of a 10 mM D-luciferin stock solution was obtained by adding the Reaction Buffer to one vial of D-luciferin (Component A). The solution was protected from light until use. A volumn of 1.62 ml of dH2O was added to a bottle containing 25 mg of DTT (Component C). The solution was then divided into ten 160 μl volumes and kept on ice until use. Additionally, a 5 mM ATP solution was prepared as an ATP standard (Component D). The reaction solution was prepared by combining the following components: A volumn of 8.9 ml dH2O, 0.5 ml 20 × Reaction Buffer (Component E), 0.1 ml 10 mM D-luciferin, and 2.5 μl of firefly luciferase 5 mg/ml stock solution. The reaction solution was mixed gently and then added to an ATP standard to plot a standard curve. The reaction solution of the same volume was added to samples, and a plate reader was used to read the results.

2.8. Nile red accumulation in mutant and gene knockout strains

A volumn of 50 μl of an overnight culture was added to 5 ml LB broth and incubated at 37°C until a cell density of OD 0.2 was reached. After centrifugation, 0.85 % saline was used to wash and resuspend the cell pellet, which was then subjected to starvation for 144 hours. Bacteria in the log phase were included as a control. The test strains were concentrated to an OD of 0.4 and stained with 5 μM of Nile red for 3 hours at 37°C. After staining, the bacteria were washed and resuspended with PBS containing 1 mM $MgCl_2$ and transferred to a 96-well black plate. Finally, the fluorescence intensity was measured by a plate reader at 560 nm and 655 nm.

2.9. Assessment of antibiotic accumulation in mutant and gene knockout strains

An inverted microscope was used to visualize the structural changes in antibiotic-tolerant persisters during various types of treatment using lasers of different wavelengths. For testing the accumulation of Bocillin FL, the excitation wavelengths and emission wavelength were set at 488 ± 10 nm and 512 ± 10 nm, respectively. For assessment of the accumulation of the Texas Red gentamicin conjugate, the excitation wavelengths and emission wavelength were adjusted to 500–600 nm and 610 nm, respectively. The EMCCD camera was used to record the fluorescence signal. The images were analyzed using ImageJ software (Fiji). Antibiotic accumulation in transporter gene knockout strains that had been subjected to nutrient starvation for six days was observed under a fluorescence microscope upon treatment with Bocillin FL or the Texas Red gentamicin conjugate. Briefly, 20 μg/ml Bocillin FL penicillin or 1.25 μg/ml Texas Red gentamicin conjugate was added to the bacterial suspension, followed by treatment with the corresponding antibiotic for 30 minutes; 0.85 % saline was used to provide a clear background for fluorescence microscopy and to wash out the surplus dye. The fluorescence intensity in different samples was recorded and compared.

2.10. Oxidative stress tolerance assay under anaerobic conditions

A 60 μL portion of an overnight culture was added to 6 ml of sterile LB broth in a test tube, followed by incubation at 37°C and shaking at 250 rpm until the OD600 reached around 0.2 (approximately 1.5 hours). Upon centrifugation and removal of the supernatant, M9 broth was used to wash and resuspend the pellet; 4 mM H2O2 was then added to the strains (Kang et al., 2013). An equal volume of paraffin was added to prevent exposure of the test organisms to air. The culture was sampled at different time points and spread onto LB plates, followed by incubation at 37°C to monitor the survival rate of the test organisms under oxidative stress.

2.11. Heat stress tolerance assay

The test strain was streaked onto an LB agar plate and incubated at 37°C overnight. A single colony was picked from the plate and inoculated into 5 ml LB broth. After overnight incubation at 37°C with shaking at 250 rpm, 60 μL of the overnight culture was inoculated into 6 ml of autoclaved LB broth in a test tube, followed by incubation at 37°C with shaking at 250 rpm until the absorbance at OD600 reached 0.2. The culture was then subjected to a temperature shift: incubated in a 25°C water bath for 10 minutes, followed by 55°C for 10 minutes, and then returned to 25°C for 10 minutes. The strains were recovered after a 4-hour incubation at 37°C with shaking at 250 rpm in a shaker (Masuda et al., 2020). A volumn of 20 ml of bacterial cultures were then added to a 96-well cell culture plate every hour, and diluted; A volumn of 10 ml of the diluted culture was spread onto an LB agar plate and incubated at 37°C. The number of colonies was counted and recorded.

3. Results

3.1. Targeted genetic changes occur 72 hours post-starvation of antibiotic persisters

We first investigated whether genetic changes occurred in bacteria during starvation. We hypothesized that physiological and cellular activities of bacteria, including nucleic acid synthesis, were reduced to an extremely low level during nutrient starvation. Therefore, any mutational changes that occurred must be due to some unknown but active and tightly-regulated mutagenesis mechanisms. In this experiment, a bacterial culture derived from a single colony of *E. coli* strains BW25113 and BW3110 was prepared and subjected to treatment as depicted in Fig. 1. Briefly, the test strains were grown to the log phase (OD600 =0.2) in the same manner as that described in a tolerance assay reported previously (Wan et al., 2021). Bacterial cells were collected at three time points, namely 0-hour, 72-hour, and 144-hour. DNA was then extracted and subjected to whole-genome sequencing. Since the entire test population was subjected to whole-genome sequencing, any mutations detectable were expected to occur frequently among the population. Mutations that only occurred in individual cells and those that occurred at a low frequency would not be detectable in this experiment.

No mutation could be detected in bacterial populations subjected to starvation for 24 hours. However, genetic changes became detectable at 72 hours. As shown in Table 1, mutations were frequently found in two genes located in close proximity to each other: *ybfD* and *rhsC*, although mutations in other genes were also identified. The most common mutation in the *ybfD* gene was the change from AAACC to CAACT, which involved two nucleotides at site 509. The corresponding amino acid change was Lys to Thr. The most common mutation in the *rhsC* gene was a change from C to T at site 539, resulting in a Ser to Asn amino acid

Table 1
Summary of mutated genes and snps identified in *E. coli* strains BW25113 and W3110 subjected to starvation for 6-days.

Strains	Time points	Repeat 1			Repeat 2		
		Nucleotide changes	Amino acid changes	Protein	Nucleotide changes	Amino acid changes	Protein
BW25113	72 h	A→G	Asn→Ser	RhsC	A→G	Asn→Ser	RhsC
					AAACC→CAACT		YbfD
	144 h	A→G	Asn→Ser	RhsC	A→G	Asn→Ser	RhsC
		AAACC→CAACT	ThrThr→LysThr	YbfD	AAACC→CAACT	ThrThr→LysThr	YbfD
W3110	72 h	AAACC→CAACT	ThrThr→LysThr	YbfD			
	144 h	AAACC→CAACT	ThrThr→LysThr	YbfD			
		G→A	Gln→*	YhhI family	A→G	Asn→Ser	RhsC
		TG→AA	Thr→Ile		AAACC→CAACT	ThrThr→LysThr	YbfD

change. Therefore, this study focused on the effect of mutations in the *ybfD* and *rhsC* genes.

3.2. Mutations in the rhsC and ybfD genes confer higher level of antibiotic tolerance

To investigate why mutations were introduced into the *rhsC* and *ybfD* genes, the functions of the products of these two genes were investigated; in addition, genes located up and downstream of these two genes were mapped and studied (Figure S1e). The results showed that genes located in close proximity to the *rhsC* gene included *ybfA, ybfB, ybfC, ybfO, ybfQ*, and the *ybfD* gene, and that they all belonged to the *ybf* cluster, but the functions of these genes have not been characterized previously (Figure S1d) (Von Mering et al., 2007). To determine the functional role of this gene cluster, a deletion mutant of each gene in this cluster was created to test whether the products of these genes play a role in stress tolerance. Also, the entire DNA fragment containing these genes was deleted to create the *E. coli* BW25113::Δ*ybf* cluster knockout strain for this purpose. These gene knockout strains were then subjected to nutrient starvation, and their antibiotic tolerance level was tested as previously described (Wan et al., 2021). All gene knockout strains tested were found to exhibit significantly lower levels of tolerance to ampicillin and gentamicin upon 6-days of starvation when compared to the wild type strain. Deletion of the *ybfB* and *ybfC* genes appeared to exhibit the strongest tolerance suppression effect; importantly, deletion of the entire *ybf* cluster resulted in further reduction in tolerance level when compared to deletion of individual genes (Figs. 2, S2). These findings indicate that the products of these genes in the wild-type strain play a certain role in mediating the onset of the antibiotic tolerance phenotype.

We next investigated whether specific mutations introduced into the *rhsC* and *ybfD* genes resulted in a higher tolerance level and survival rate (Fig. 3). The corresponding mutants were created by the Flp-FRT recombination technology. Assessment of the survival rate of these mutants upon prolonged starvation and treatment with high doses of gentamicin showed that the population size of *E. coli* BW25113::*ybfD*, *rhsC*, as well as the *ybfDrhsC* double mutant, decreased from 10^8–10^4 per ml. This rate of population size reduction was much slower than that of the wild type, whose population size was found to reduce to 10^2 per ml upon treatment for six days. This observation indicates that the mutants exhibited significantly higher survival fitness during starvation and antimicrobial treatment when compared to the wild type. On the other hand, although no apparent difference in the tolerance level between the wild type and mutant strains was observed during ampicillin treatment for six days, a higher survival rate was observed in the *E. coli*

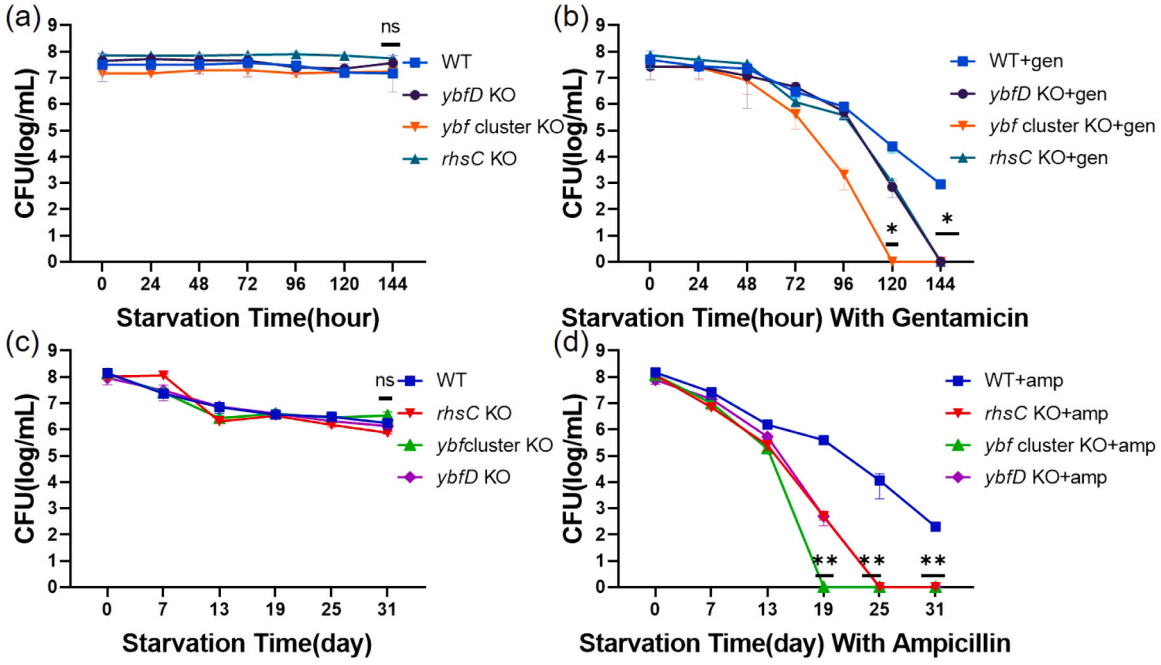

Fig. 2. Survival levels of gene deletion strains. Survival levels of the *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 6-day starvation(a). Survival levels of the *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 6-day starvation with gentamicin treatment(b). Survival levels of the *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 31-day starvation(c). Survival levels of the *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 31-day starvation with ampicillin treatment(d). Each value is presented as the mean, and error bar indicates SD. * , $P < 0.05$, * *, $P < 0.01$, * ** , $P < 0.001$, ns, $P \geq 0.05$.

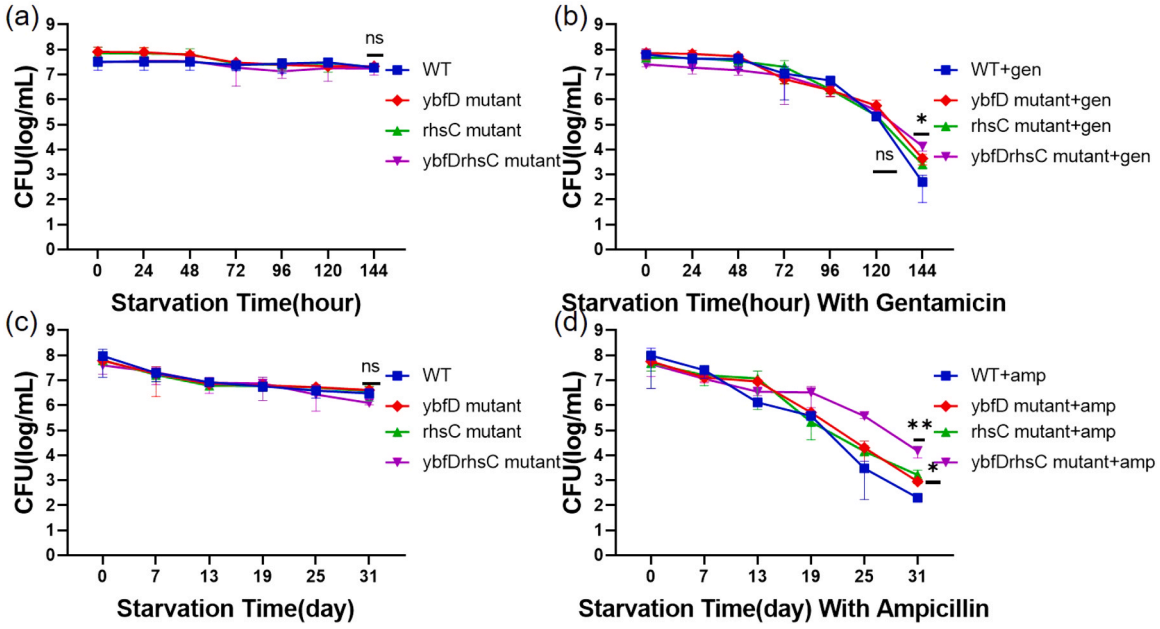

Fig. 3. Survival levels of mutants in which specific mutations were introduced. Survival levels of the *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 31-day starvation (a) Survival levels of the *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 31-day starvation withgentamicin treatment(b) Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* as well as the *ybfDrhsC* double mutants under 31-day starvation(c) Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* as well as the *ybfDrhsC* double mutants under 31-day starvation with ampicillin treatment(d). Each value is presented as the mean, and error bar indicates SD. * , $P < 0.05$, * *, $P < 0.01$, * ** , $P < 0.001$, ns, $P \geq 0.05$.

BW25113::*ybfD* and *rhsC* mutants when the treatment was prolonged to one month, after which a population size of 10^3 per ml was still detectable. The survival of the *E. coli* BW25113::*ybfDrhsC* double mutant was even higher, with a population size of 10^5 per ml being recorded after treatment for one month with ampicillin. This finding indicates that, although the products of the *ybf* gene cluster do not appear to play a major role in mediating the maintenance of bacterial antibiotic tolerance, mutations in at least two genes in this cluster, namely *ybfD* and *rhsC*, render them able to significantly enhance the survival fitness of bacteria upon antimicrobial treatment under prolonged starvation conditions. We next created the rescue strains of the mutants and found that the survival level of the rescue strains reverted back to that of the wild type (Figure S3), confirming that mutations in the *ybfD* and *rhsC* genes contribute to enhanced survival fitness and antibiotic tolerance in bacteria upon prolonged starvation.

3.3. Mutations in the ybfD gene significantly enhance the activity of the RhsC protein

To investigate why the *rhsC* and *ybfD* genes need to be mutated upon encountering starvation for a prolonged period, the possible relationship between the wild type and mutant YbfD and RhsC proteins was examined. The C-terminal end of the RhsC protein exhibited structural similarity to the VipE protein of *Legionella pneumophila* (Vazquez-Lopez and Navarro-Garcia, 2020), which plays a role in vesicular traffic (Shohdy et al., 2005). Furthermore, the RhsC protein was found to play a role in toxin delivery into plant cells as its sequence is closely linked to a component of the T6SS genes (Koskiniemi et al., 2013). We therefore hypothesized that RhsC was a transmembrane protein, and that the mutant YbfD protein may help enhance the function of the RhsC protein. Based on such circumstantial evidence, we next investigated whether the YbfD protein plays a role in regulating the expression of the *rhsC* gene by performing quantitative RT-PCR in the wild type, the *E. coli* BW25113::Δ*ybfD* knockout strain, and the *E. coli* BW25113::*ybfD* mutant, followed by the measurement and comparison of the expression level of the *rhsC* gene in these three samples. As *ybfB* was also predicted to be a transporter gene and related to *ybfD* and *rhsC* (Figure S1d), it was also included in this assay, although the killing curve and fluorescent microscopy results of it is not significant. The results showed that the expression of the *rhsC* and *ybfB* gene was down-regulated in the *E. coli* BW25113::Δ*ybfD* deletion strain, indicating that YbfD plays a role in mediating the production of the RhsC protein. Interestingly, the expression level of *rhsC* and *ybfB* was even lower in the *E. coli* BW25113::*ybfD* mutant (Figure S1b); however, the tolerance level of the *E. coli* BW25113::*ybfD* mutant was found to increase when compared to the wild type. These observations suggest that the mutation in the *ybfD* gene could significantly enhance the activity of the RhsC protein and suppress the expression level of the protein to conserve cellular materials and energy under adverse environmental conditions. In other words, the mutant YbfD protein may play a dual role in up-regulating the expression of the *rhsC* gene and directly enhancing the activity of the RhsC protein. This theory needs to be tested in future works.

3.4. The ybf gene cluster plays a role in efflux function in bacterial antibiotic persisters

We found that deletion of genes in the *ybf* gene cluster also did not affect the ATP level in persisters (Figure S4a). Similarly, the membrane potentials of all knockout strains were comparable to the wild-type strain (Figure S4b). Assessment of the level of antibiotic accumulated in these strains, however, showed that Bocillin FL accumulated at a higher level in the *E. coli* BW25113::Δ*ybfD*, Δ*ybfO*, Δ*ybfQ*, Δ*rhsC*, and Δ*ybf* cluster knockout strains (Fig. 4**a, d, S5**), exhibiting a fluorescence intensity of 4×10^6, which was about double that of the *E. coli* BW25113::Δ*ybfA*, Δ*ybfB*, Δ*ybfC* gene knockout strains (2×10^6). Likewise, Texas red gentamicin conjugates were also found to accumulate at a higher level in the *E. coli* BW25113::Δ*ybfD*, Δ*ybfO*, Δ*ybfQ*, and Δ*rhsC* gene knockout strains (Fig. 4**b, e, S6**), with a fluorescence intensity of 6×10^5 being recorded; the fluorescence level in the *E. coli* BW25113::Δ*ybf* cluster knockout strain was even as high as 8×10^5. However, the fluorescence intensity of the *E. coli* BW25113::Δ*ybfA*, Δ*ybfB*, Δ*ybfC* knockout strains remained the same as the wild type (4×10^5). Since RhsC was predicted to be a component of the secretion system T6SS, efflux activity of the knockout strains was tested using Nile

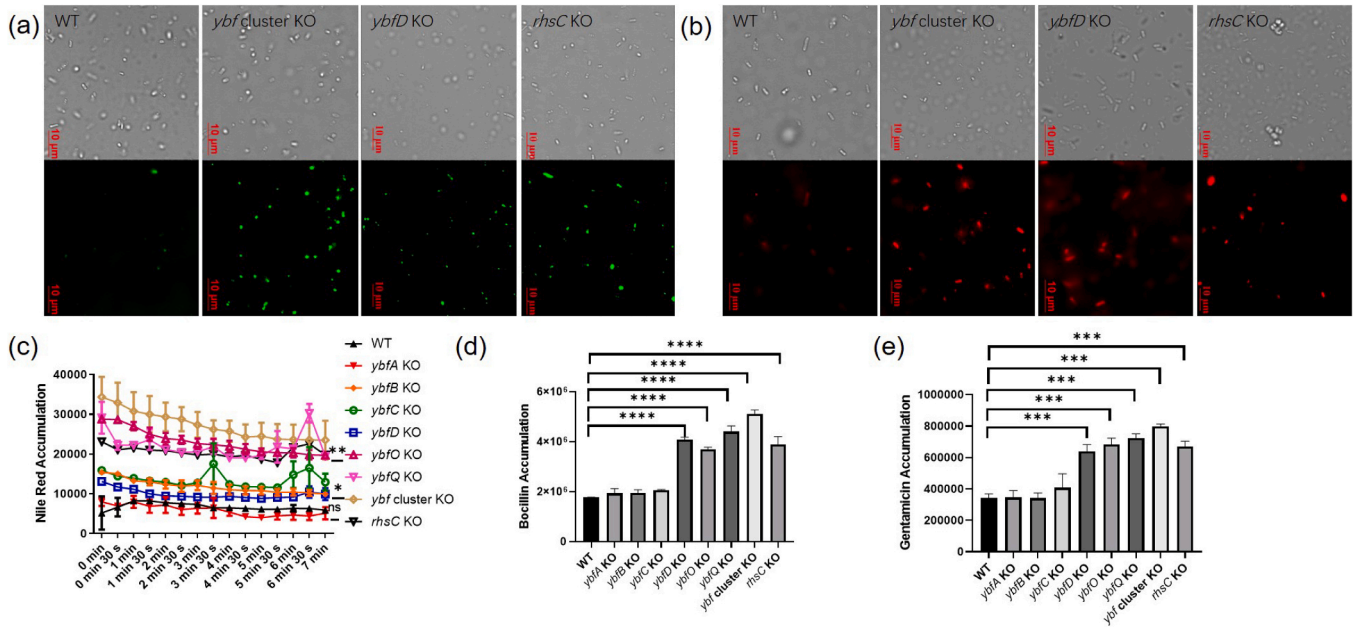

Fig. 4. Efflux pump activity of gene deletion strains. Bocillin FL accumulation in *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 6-day starvation (a). Texas red gentamicin conjugates accumulation in *E. coli* BW25113::Δ*ybfD*, Δ*rhsC*, and Δ*ybf* cluster knockout strains upon 6-day starvation (b). Nile red accumulation of *E. coli* BW25113::Δ *ybfA*, Δ*ybfB*, Δ*ybfC*, Δ*ybfD*, Δ*ybfO*, Δ*ybfQ*, Δ*ybf* cluster and Δ*rhsC* knockout strains upon 6-day starvation (c). Bocillin FL fluorescence intensity of *E. coli* BW25113::Δ*ybfA*, Δ*ybfB*, Δ*ybfC*, Δ*ybfD*, Δ*ybfO*, Δ*ybfQ*, Δ*ybf* cluster and Δ*rhsC* knockout strains upon 6-day starvation(d). Texas red fluorescent intensity of *E. coli* BW25113::Δ*ybfA*, Δ*ybfB*, Δ*ybfC*, Δ*ybfD*, Δ*ybfO*, Δ*ybfQ*, Δ*ybf* cluster and Δ*rhsC* knockout strains upon 6-day starvation (e). Each value is presented as the mean, and error bar indicates SD. * , $P < 0.05$, * *, $P < 0.01$, * ** , $P < 0.001$, ns, $P \geq 0.05$.

red as a substrate (Bohnert et al., 2010). Measurement of the degree of accumulation of the fluorescent Nile red dye in the gene knockout strains upon starvation for six days (Fig. 4**c**) showed that the fluorescence intensity of the *E. coli* BW25113::Δ*ybfA* knockout strain was the same as the wild type (lower than 10,000) (Fig. 4**c**); however, significant accumulation of Nile Red was recorded in other gene

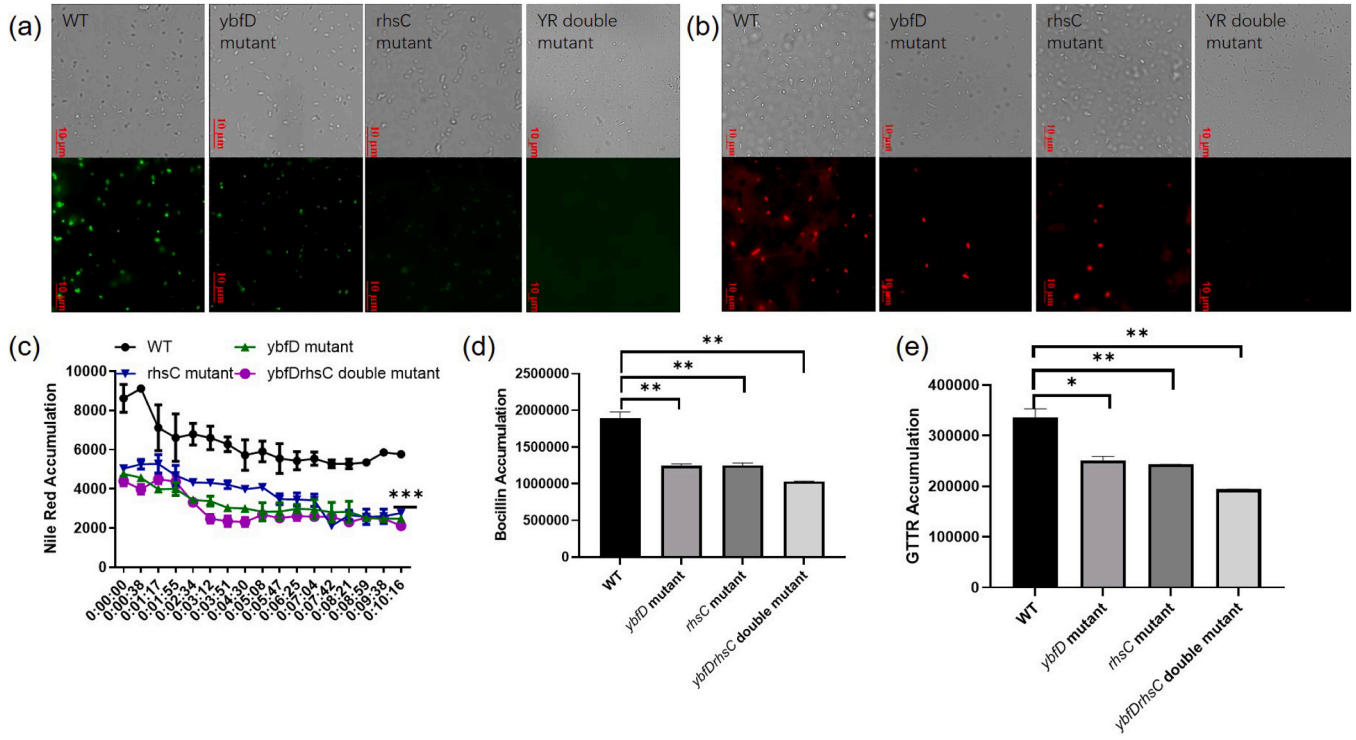

Fig. 5. Efflux pump activity of mutants in which specific mutations were introduced. Bocillin FL accumulation in *E. coli* BW25113::*ybfD*, *rhsC*, and *ybfDrhsC* mutants upon 6-day starvation (a). Texas red gentamicin conjugates accumulation in *E. coli* BW25113::*ybfD*, *rhsC*, and *ybfDrhsC* mutants upon 6-day starvation (b). Nile red accumulation of *E. coli* BW25113::*ybfD*, *rhsC*, and *ybfDrhsC* mutants upon 6-day starvation(c). Bocillin FL fluorescent intensity of *E. coli* BW25113::*ybfD*, *rhsC*, and *ybfDrhsC* mutants upon 6-day starvation(d). Texas red fluorescent intensity of *E. coli* BW25113::*ybfD*, *rhsC*, and *ybfDrhsC* mutants upon 6-day starvation(e). Each value is presented as the mean, and error bar indicates SD. * , $P < 0.05$, * *, $P < 0.01$, * ** , $P < 0.001$, ns, $P \geq 0.05$.

knockout strains. Among them, the *E. coli* BW25113::*ΔybfB, ΔybfC*, and *ΔybfD* knockout mutants exhibited a significantly higher fluorescence level than the wild-type strain (around 15,000); the fluorescence level of the *E. coli* BW25113::*ΔrhsC*, *ΔybfO*, and *ΔybfQ* knockout strains was even higher (20,000–30,000), whereas that of the *E. coli* BW25113::*Δybf* cluster knockout strain intensity was as high as 35,000. These data show that members of the *ybf* gene cluster encode proteins that play a role in mediating active efflux in antibiotic persisters.

To investigate the cellular mechanisms underlying the elevated survival rate of the *E. coli* BW25113::*ybfD* and *rhsC* mutants upon starvation and antimicrobial treatment, efflux activity of the single and double mutants created artificially in our laboratory was measured. The intensity of Nile red accumulated in the *E. coli* BW25113::*ybfD*, *rhsC*, and *ybfDrhsC* mutants was around 5000, which was almost half that of the wild-type, indicating that efflux activity had significantly increased in these mutants. Determination of the degree of antibiotic accumulation in the *E. coli* BW25113::*ybfD* and *rhsC* mutants showed that Bocillin FL and the Texas red gentamicin conjugate accumulated at a significantly lower rate, as the fluorescence intensity of these two conjugates recorded decreased by 50 % and 33 %, respectively, when compared to that of the wild-type strain. Based on these findings, we conclude that mutations in the *ybfD* and *rhsC* genes may result in increase in efflux activity of persisters (Fig. 5).

3.5. Mutations in the ybfD and rhsC genes confer higher antibiotic tolerance induced by heat and oxidative stress

As specific mutations were found to enhance the antibiotic tolerance level and efflux activity of *E. coli* strain BW25113 under starvation stress, we further tested whether such mutations also conferred tolerance to other stresses, or antibiotic tolerance induced by other stresses. Heat stress is another condition that induces tolerance formation; in this study, bacteria were subjected to heat shock at 55°C for 10 minutes. The test organisms were incubated at 25°C for 10 minutes before and after the heat shock. The bacteria were allowed to recover at 37°C for 4 hours. Upon treatment with high doses of ampicillin and gentamicin, the bacteria of all the test groups remained viable, indicating that antibiotic tolerance had formed (Fig. 6 **a, b, c**). The survival rate of the mutants was the same as that of the wild-type upon ampicillin treatment. Upon gentamicin treatment, the survival rate of persisters of the *E. coli* BW25113::*rhsC* mutant was the same as that of the wild-type strain and were completely eradicated within 2 hours, but that of persisters of the *E. coli* BW25113::*ybfD* and *ybfDrhsC* strains was significantly higher, and the persisters of these two strains were only completely killed after

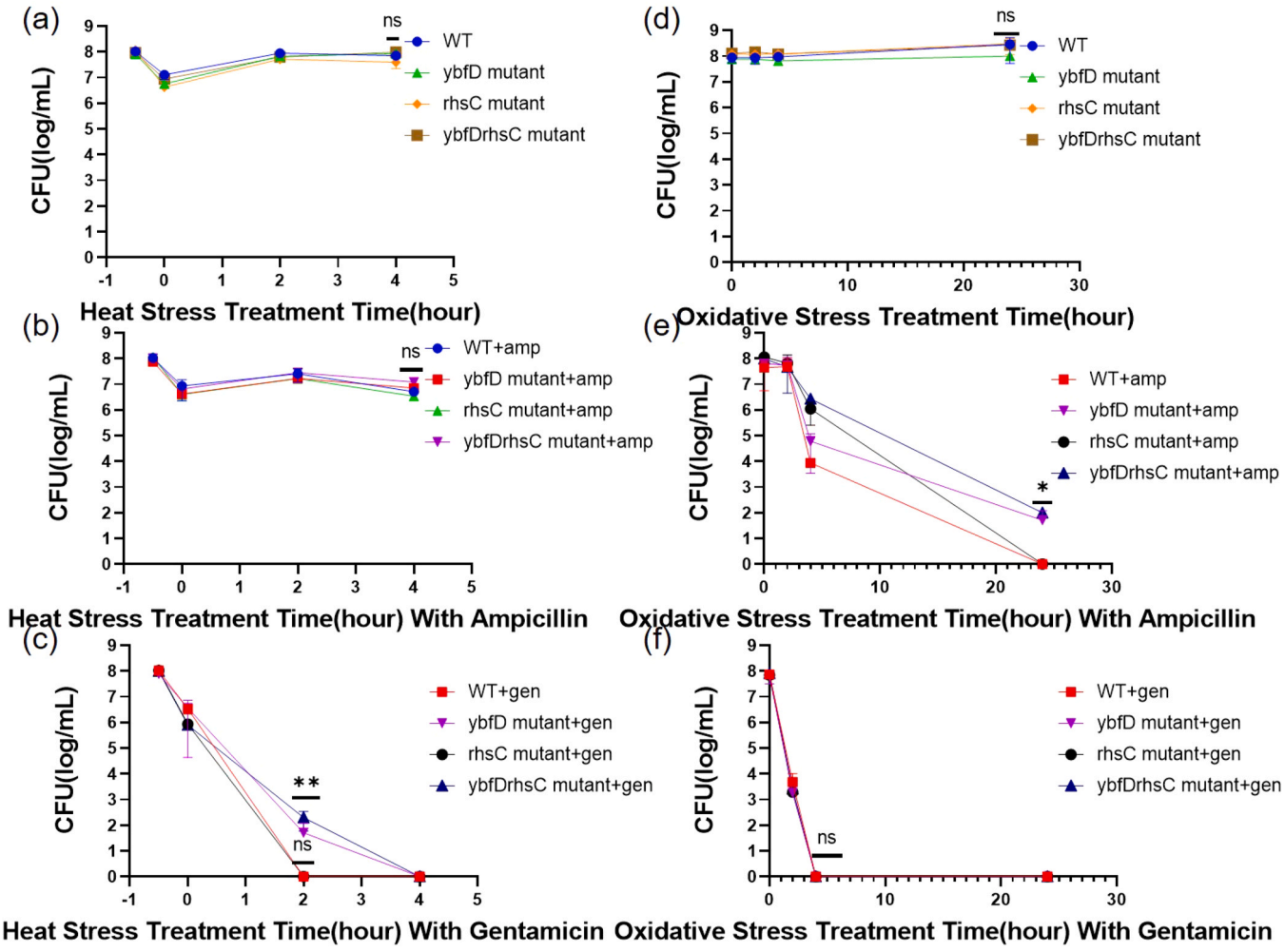

Fig. 6. Survival levels of strains in which specific mutations were introduced. Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* mutants as well as *ybfDrhsC* double mutants under heat stress(a). Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* mutants as well as *ybfDrhsC* double mutants under heat stress with ampicillin treatment (b). Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* mutants as well as *ybfDrhsC* double mutants under heat stress with gentamicin treatment (c). Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* as well as *ybfDrhsC* double mutants under oxidative stress(d). Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* as well as *ybfDrhsC* double mutants under oxidative stress with ampicillin treatment(e). Survival levels of *E. coli* BW25113::*ybfD*, *rhsC* as well as *ybfDrhsC* double mutants under oxidative stress with gentamicin treatment(f). Each value is presented as the mean, and error bar indicates SD. * , $P < 0.05$, * *, $P < 0.01$, *** , $P < 0.001$, ns, $P \geq 0.05$.

4 hours of treatment with gentamicin. This finding shows that mutations in the *ybfD* gene could at least enhance the survival level of gentamicin persisters.

Oxidative stress is another important category of stress conditions that can induce formation of bacterial antibiotic tolerance. In this study, we used hydrogen peroxide (H_2O_2) to create oxidative stress; paraffin oil was added to the culture tube to prevent exposure to air and create an anaerobic condition. Upon prolonged gentamicin treatment, persisters of all test groups were completely eradicated within 4 hours, and bacterial regrowth in nutrient broth was not observed after incubation at 37°C for 24 hours. In the ampicillin treatment assay, however, strains carrying mutations in the *rhsC* gene, including the *E. coli* BW25113::*rhsC* single-gene mutant and the *ybfDrhsC* double mutant, exhibited a significantly higher survival rate; a population size of 10^2 per ml of these two mutants remained detectable after 24-hour treatment, whereas the wild-type strain and the *E. coli* BW25113::*ybfD* single mutant were completely eradicated in less than 24 hours. (Fig. 6e). This finding indicates that mutations in the *rhsC* and *ybfD* genes act synergistically to confer a higher degree of tolerance to the β-lactam drugs.

4. Discussion

Bacteria utilize a wide range of cellular mechanisms to combat environmental stress and enhance survival fitness. Understanding the genetic and physiological basis of such mechanisms would enable the development of novel antimicrobial strategies that act by suppressing bacterial stress responses. Such strategies may target all types of bacterial pathogens regardless of their virulence level and antibiotic susceptibility. Our recent studies showed that bacteria can undergo a range of physiological changes that allow them to endure environmental stress for a certain period of time (Wan et al., 2023). However, these changes, which involve active efflux to exude toxic metabolites and antimicrobial drugs, are actually energy demanding and require maintenance of the transmembrane proton motive force (Wan et al., 2021). It is almost impossible for bacterial persisters to maintain such functions for a prolonged period if nutrient starvation conditions persist. Hence, persisters may need to utilize other means to prolong survival under stress. In this work, we found that persisters started to undergo mutational changes in specific genes, and that the efflux activity and tolerance level of the mutated strains increased significantly. Interestingly, we also found that the expression level of one of the mutated proteins, RhsC, actually decreased when mutations occurred in the *ybfD* gene (Fig S1). It appears that bacterial persisters need to improve efflux activity by altering the structure of one or more efflux pumps so that efflux activity can be maintained with a much smaller number of proteins under prolonged starvation conditions. In a previous study, we showed that protein synthesis activity was still active in persisters upon encountering long-term starvation but, nevertheless, the level decreased gradually. Apparently, enhancing the activity of specific proteins and strengthening the structure of the protein can help solve the problem of having to continue to produce efflux pumps or transporter proteins under adverse growth conditions. We hypothesize that bacteria also need to undergo specific genetic changes to enable the structures of various other important cellular proteins to alter into a stress-resistant state when adverse environmental conditions persist for over a certain period of time. In this work, we confirm that bacteria could start to undergo active genetic changes in specific genes upon encountering starvation for three days (Table 1). This finding is intriguing for several reasons: (1) mutational changes only occurred when nutrient starvation conditions persisted for more than six days, indicating that some hitherto unknown mechanisms for sensing the degree of starvation stress and mutagenesis exist in bacterial antibiotic persisters, (2) mutations only occurred at specific sites, suggesting that site-specific mutagenesis mechanisms were involved, (3) cellular activities in bacteria under prolonged starvation stress are expected to be switched to a minimal level, hence the physiological basis of the mutagenesis process concerned needs to be delineated. Currently, a number of cellular mechanisms that enable bacteria to undergo genetic changes upon encountering environmental stresses have been identified. These include switching to the use of error-prone polymerases to increase the probability of generating mutants that can survive against specific stresses. More recently, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system has been identified in bacteria. This system was found to enable bacteria to destroy genetic materials of invading bacteriophages and alter the genetic composition to enhance survival fitness under adverse environmental conditions (Le Gratiet et al., 2021). However, it is not clear whether bacteria can utilize the CRISPR system or other cellular mechanisms to undergo site-specific mutagenesis during nutrient starvation. In this work, we observed the occurrence of site-specific mutagenesis during starvation but have not identified the mechanism concerned. Currently, the CRISPR technique is being utilized for genetic editing purposes, which can potentially cure genetic diseases. It is necessary to investigate whether the phenomenon of site-specific mutagenesis observed in this work involves an unknown mechanism.

Introduction of mutations into such genes therefore makes sense, as a mutant YbfD protein may contribute to higher genetic plasticity and a higher chance of survival under adverse conditions. The function of the YbfD protein has not been clearly elucidated and needs to be investigated in the future. On the other hand, the RhsC protein, the product of the *rhsC* gene in which mutations emerged in bacterial persisters that have encountered prolonged starvation, was predicted to be a component of the type VI secretion system and functionally related to the VacJ/Yrb ABC (ATP-binding cassette) transport system, a proposed phospholipid transporter involved in outer membrane vesicle formation (Roier et al., 2016). This finding is consistent with that of our previous studies, which showed that bacterial antibiotic persisters had to maintain the tolerance phenotype by up-regulating efflux activities (Wan et al., 2021).

It is intriguing that mutations in the *ybfD* gene recorded in this study mostly involved a Lys to Thr amino acid substitution, whereas in the *rhsC* gene, a Ser to Asn change was often found. Mutations in these two genes, both located in the *ybf* gene cluster, often occur simultaneously. Introduction of these two mutations into the *E. coli* BW25113 wild-type strain to test their effect on the survival fitness of persisters showed that organisms which were subjected to prolonged starvation and have acquired such mutations survived significantly better than the wild-type strain upon treatment with both gentamicin and ampicillin. Importantly, the survival level of the *E. coli* BW25113::*ybfDrhsC* double mutant was even higher than that of the single mutants under 1-month starvation and ampicillin treatment (Fig. 3). Mutations in the *rhsC* and *ybfD* genes were found to result in a decrease in antibiotic and Nile red accumulation (Fig. 5). We confirmed that the mutant RhsC protein exhibited elevated efflux pump and secretion activities to enhance the survival fitness of bacterial cells subjected to prolonged starvation and maintain the antibiotic tolerance phenotype, despite the fact that the expression level of the mutated *rhsC* gene was lower than the wild type as discussed above. Another important discovery of this work is that the mutant YbfD could regulate the expression level of the *rhsC* gene (Fig S1). The functional relationship of the YbfD and RhsC proteins needs to be investigated to determine if these proteins can actually interact with each other to enhance survival fitness of bacteria under adverse environmental conditions. Importantly, persisters of the *E. coli* BW25113::*ybfDrhsC* double mutants also exhibited higher survival levels under oxidative stress and heat stress (Fig. 6). It is not clear how a structurally altered transporter can contribute to a higher level of oxidative and heat stress. Nevertheless, these findings appear to suggest that the *ybfD* and *rhsC* mutations that were actively introduced in bacteria which have experienced prolonged starvation conferred not only antibiotic tolerance but also significantly higher survival fitness of bacteria under extreme environmental conditions. It should be noted that the survival-enhancing effects observed in this study were attributed to single or double mutations in the *ybfD* and *rhsC* genes introduced artificially. We

envisage that naturally selected mutants which contain multiple mutations in the genome exhibited an even higher level of stress tolerance. To conclude, the two major findings of this work, namely a starvation-induced site-directed mutagenesis mechanism and the YbfD and RhsC-mediated stress tolerance response, need to be further studied to better understand how bacteria cope with prolonged starvation stress.

It should be noted that this work involved investigation of an important scientific and clinical phenomenon which has puzzled both basic scientists and clinicians for over 80 years. This phenomenon is antibiotic tolerance, which can be distinguished from antibiotic resistance by the fact bacterial tolerant cells are unresponsive to antibiotics, but cannot grow in the presence of such drugs, whereas antibiotic resistant strains are known to have acquired genetic mutations or exogenous resistant elements such as plasmids, and can evade the antimicrobial actions of antibiotics and even grow in the presence of antimicrobial drugs. Antibiotic tolerant bacterial cells are now known to be responsible for causing chronic and recurrent infection. This finding represents a significant breakthrough in investigation of the underlying mechanism of bacterial antibiotic tolerance. The efflux genes in which tolerance-enhancing mutations are introduced during starvation or other adverse environmental conditions may be regarded as good drug targets for development of new strategies to eradicate bacterial antibiotic tolerance sub-populations.

CRediT authorship contribution statement

Chen Sheng: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Tang Yang:** Data curation. **Chan Edward Wai-Chi:** Writing – review & editing. **Ye Lianwei:** Formal analysis. **Zheng Jiaqi:** Data curation. **WAN Yingkun:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

No.

Acknowledgement

The work was supported by the Guangdong Major Project of Basic and Applied Basic Research (2020B0301030005) and grants provided by the Research Grant Council of Hong Kong Government (T11–104/22-R and R1011–23).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2025.128106.

Data Availability

The data that has been used is confidential.

References

Bhamre, S., Gadea, B.B., Koyama, C.A., White, S.J., Fowler, R.G., 2001. An aerobic recA-, umuC-dependent pathway of spontaneous base-pair substitution mutagenesis in Escherichia coli. Mutat. Res. /Fundam. Mol. Mech. Mutagen. 473 (2), 229–247.

Bigger, J.W., 1944. The bactericidal action of penicillin on Staphylococcus pyogenes. Ir. J. Med. Sci. (1926-1967) 19 (11), 553–568.

Bjedov, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman, M., Taddei, F., Matic, I., 2003. Stress-induced mutagenesis in bacteria. Science 300 (5624), 1404–1409.

Bohnert, J. r A., Karamian, B., Nikaido, H., 2010. Optimized Nile Red efflux assay of AcrAB-TolC multidrug efflux system shows competition between substrates. Antimicrob. Agents Chemother. 54 (9), 3770–3775.

Bull, H.J., Lombardo, M.-J., Rosenberg, S.M., 2001. Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. Proc. Natl. Acad. Sci. 98 (15), 8334–8341.

Cairns, J., Overbaugh, J., Miller, S., 1988. The origin of mutants. Nature 335 (6186), 142–145.

Dunai, A., Spohn, R., Farkas, Z., Lazar, V., Györkei, Á., Apjok, G., Boross, G., Szappanos, B., Grezal, G., Farago, A., 2019. Rapid decline of bacterial drug-resistance in an antibiotic-free environment through phenotypic reversion. Elife 8, e47088.

Gressel, J., Levy, A.A., 2010. Stress, mutators, mutations and stress resistance. Abiotic Stress Adapt. Plant.: Physiol., Mol. Genom. Found. 471–483.

Kang, A., Tan, M.H., Ling, H., Chang, M.W., 2013. Systems-level characterization and engineering of oxidative stress tolerance in Escherichia coli under anaerobic conditions. Mol. BioSyst. 9, 285–295.

Koskiniemi, S., Lamoureux, J.G., Nikolakakis, K.C., t'Kint de Roodenbeke, C., Kaplan, M. D., Low, D.A., Hayes, C.S., 2013. Rhs proteins from diverse bacteria mediate intercellular competition. Proc. Natl. Acad. Sci. 110 (17), 7032–7037.

Lagage, V., Chen, V., Uphoff, S., 2022. Adaptation delay causes a burst of mutations in bacteria responding to oxidative stress. bioRxiv.

Le Gratiet, T., Le Marechal, C., Devaere, M., Chemaly, M., Woudstra, C., 2021. Exploration of the diversity of clustered regularly interspaced short palindromic repeats-cas systems in Clostridium novyi sensu lato. Front. Microbiol. 12, 711413.

Lenski, R.E., Mittler, J.E., 1993. The directed mutation controversy and neo-Darwinism. Science 259 (5092), 188–194.

Luria, S.E., Delbrück, M., 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28 (6), 491.

Maenhaut-Michel, G., Blake, C.E., Leach, D.R., Shapiro, J.A., 1997. Different structures of selected and unselected araB-lacZ fusions. Mol. Microbiol. 23 (6), 1133–1145.

Masuda, Y., Sakamoto, E., Honjoh, K.-i., Miyamoto, T., 2020. Role of toxin-antitoxin-regulated persister population and indole in bacterial heat tolerance. Appl. Environ. Microbiol. 86, e00935–00920.

Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O., Klug, L., Gadermaier, B., Weinzerl, K., Prassl, R., 2016. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. Nat. Commun. 7 (1), 10515.

Schmitz, M., Querques, I., 2024. DNA on the move: mechanisms, functions and applications of transposable elements. FEBS Open Bio 14 (1), 13–22.

Shohdy, N., Efe, J.A., Emr, S.D., Shuman, H.A., 2005. Pathogen effector protein screening in yeast identifies Legionella factors that interfere with membrane trafficking. Proc. Natl. Acad. Sci. 102 (13), 4866–4871.

Sulaiman, J.E., Lam, H., 2021. Evolution of bacterial tolerance under antibiotic treatment and its implications on the development of resistance. Front. Microbiol. 12, 617412.

Tabata, S., Higashitani, A., Takanami, M., Akiyama, K., Kohara, Y., Nishimura, Y., Nishimura, A., Yasuda, S., Hirota, Y., 1989. Construction of an ordered cosmid collection of the Escherichia coli K-12 W3110 chromosome. J. Bacteriol. 171 (2), 1214–1218.

Vazquez-Lopez, J., Navarro-Garcia, F., 2020. In silico analyses of core proteins and putative effector and immunity proteins for T6SS in enterohemorrhagic E. coli. Front. Cell. Infect. Microbiol. 10, 195.

Von Mering, C., Jensen, L.J., Kuhn, M., Chaffron, S., Doerks, T., Krüger, B., Snel, B., Bork, P., 2007. STRING 7—recent developments in the integration and prediction of protein interactions. Nucleic Acids Res. 35 (_1), D358–D362.

Wan, Y., Wai Chi Chan, E., Chen, S., 2023. Maintenance and generation of proton motive force are both essential for expression of phenotypic antibiotic tolerance in bacteria. Microbiol. Spectr. 11 (5) e00832-00823.

Wan, Y., Wang, M., Chan, E.W.C., Chen, S., 2021. Membrane transporters of the major facilitator superfamily are essential for long-term maintenance of phenotypic tolerance to multiple antibiotics in E. coli. Microbiol. Spectr. 9 (3) e01846-01821.

Wang, M., Chan, E.W.C., Wan, Y., Wong, M.H.-y, Chen, S., 2021. Active maintenance of proton motive force mediates starvation-induced bacterial antibiotic tolerance in Escherichia coli. Commun. Biol. 4 (1), 1068.

Yang, Y., Yu, Q., Wang, M., Zhao, R., Liu, H., Xun, L., Xia, Y., 2022. Escherichia coli BW25113 competent cells prepared using a simple chemical method have unmatched transformation and cloning efficiencies. Front. Microbiol. 13, 838698.