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# Accelerated evolution of bacterial antibiotic resistance through early emerged stress responses driven by photocatalytic oxidation

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ABSTRACT

Here, we found that the photocatalysis (PC) could elicit bacterial stress response that transiently mediate some mechanism such as efflux pumps, biofilm formation, increased mutation rates (3–5-fold changes), leading to the improved antibiotic-tolerance. The time needed to inactivate 99 % of bacteria increased from 30 to 150 min for polymyxin (PB), 55 to 135 min for tetracycline (TET), 14–40 min for ciprofloxacin (CIP), 22–35 min for streptomycin (SM), and 29–45 min for azithromycin (AZI). Afterwards, the bacteria with higher antibiotic-tolerance could evolve to antibiotic-resistance faster with subsequent antibiotic selection. The fold change of antibiotic-resistance level was up to 32 for PB, 32 for TET, 16 for CIP, 16 for SM and 4 for AZI, which are higher than those of normal bacteria. Our results suggest that, besides antibiotic, unfavorable environmental factors such as PC might give assistance to development of bacterial antibiotic-resistance potentially.

## 1. Introduction

The occurrence and proliferation of antibiotic-resistant bacteria is a serious threat to public health, which was unanimously ascribed to the extensive overuse or misuse of antibiotics in the clinic as well as residual antibiotics in the environmental system through discharge or excretion [1,2]. Two main processes have been proposed for the development of bacterial antibiotic resistance: (a) clinical antibiotic application and (b) environmental antibiotic exposure. Conventionally, the struggle against the development of antibiotic resistance has mainly taken place in the clinic and in the community [3,4]. For environmental processes, based on which antibiotics are widespread at varying concentrations [5,6], antibiotic resistance can evolve through the sequential accumulation of multiple antibiotics fitted variants [7].

Except that antibiotics have a role in the development of bacterial antibiotic resistance, bacteria may encounter a myriad of other unfavourable or lethal threats in the natural environment or anthropogenically, which could influence the bacterial stress response system and impact corresponding gene expression patterns and cell physiology in ways that can also play a fatal role in antibiotic resistance development [8,9]. Through the methodology of gene knockout or the construction of strains overexpressing some stress response-related genes (stress genes), previously published studies have illustrated the necessity of the existence of stress genes, which compose a part of bacterial inherent antibiotic resistance [10]. Additionally, the relationships between antibiotic resistance and stress responses, which are related to oxidative stress, envelope stress and other mechanisms, have already been suggested [11,12]. However, direct evidence of the stress responses that contribute to the occurrence of bacterial antibiotic resistance is not yet available. Thus, exactly how the “stressors” affect the development of bacterial antibiotic resistance has remained obscure until recently.

Photocatalysis (PC), as an extreme oxidative stimulus, has great advantages in wastewater disinfection and treatment as compared to the conventional chlorine oxidation and UV irradiation, which cause no distinct decrease of antibiotic-resistance gene (ARG) levels [13,14]. PC could cause strong oxidative pressure to bacteria and destroy their cell membrane, leading leakage of bacterial intracellular substances [15]. Additionally, the leaking substance such as protein and genetic materials as well as ARG could be further degraded, which could eliminate problem of antibiotic-resistance thoroughly [16]. For example, PC used by immobilized $TiO_2$ with LEDs could remove micropollutants and ARG in urban wastewater and surface water [17]. Heterogeneous PC using UVA-LEDs could eliminate both ARB and ARG [18]. Besides, the

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modification of some catalyst or materials loading such as photocatalytic reactive ultrafiltration membrane could also found to remove ARB and ARG from wastewater effluent [19], which shown strong feasibility in photocatalytic application in wastewater treatment and disinfection as well as antibiotic resistance problem. Some other study also show that grapheme-based $TiO_2$ composite photocatalysts could removal antibiotics, ARB as well as their associated genes [20]. Immobilized cerium-doped zinc oxide as photocatalyst could also be used for degradation of antibiotics and ARB [21]. Besides, some studies also assessed the effect of thin-film PC inactivation on the antibiotic resistance, where 99 % bacteria could be inactivated within 180 min [22]. It has been also reported that grapheme-based $TiO_2$ &Ag composite photocatalysts could reduce the bacterial antibiotic resistance to tetracycline (TET) and promote horizontal transfer of ARGs under the solar irradiation [23]. However, it is still controversial topic whether the PC could alter bacterial antibiotic resistance, and the resistance to different antibiotic such as TET and vancomycin (VAN) do significantly changed by certain time of PC [24,25]. Besides, the oxidation posed by PC can also cause adverse effects to antibiotic-resistance problem by facilitate persistence of antibiotic-resistance bacteria (ARB) when intensity of PC are as low as sublethal level [26]. Based on its strong ability to pose oxidation pressure mediated by reactive oxygen species (ROSs) [27,28], bacteria would arouse stress responses when encountering the PC interface. Notably, the stress responses that occur under exposure to PC might also have some relationship with bacterial antibiotic susceptibility. However, information about how bacteria respond to PC and how this type of anthropogenic adverse factor will influence the development of antibiotic resistance is still unclear.

Herein, PC was considered as one of the unfavourable threat conditions to study its influence on the bacterial stress responses and the development of bacterial antibiotic resistance. For this purpose, two strategies were employed to investigate the development of antibiotic resistance in the ancestral antibiotic-susceptible strain *E. coli* DH5α. One strategy is through lethal-PC induction, where the bacteria is alternately exposed to PC oxidation technology and nutrient Luria Broth (LB); another strategy is through cross-induction, where the bacteria are alternately exposed to PC oxidation technology and selected antibiotics.

## 2. Materials and methods

### 2.1. Preparations for bacterial strains, culture conditions, PC experimental design and antibiotics

Parental *E. coli* DH5α (China General Microbiological Culture Collection Center, China) was used as an antibiotic-susceptible strain. The antibiotic-resistant bacteria, namely *E. coli* DH5α (CTX), *E. coli* DH5α (PB), and *E. coli* DH5α (CTX, PB), were obtained from *E. coli* DH5< by introducing a plasmid containing a cefotaxime-resistant gene, a polymyxin-resistant gene and both of these two resistant genes, respectively. All bacterial strains were incubated in LB nutrient broth (Sangon, China) at 37 ˚C overnight with shaking. The PC reactor was a 250 ml of Quartz beaker. To check the effect of PC, four UVA LEDs lamps (total of 12 W) with a maximum emission at 365 nm were used as light resources and $TiO_2$ (Degussa P25, Germany) was applied as semiconductor photocatalyst with final concentration of 0.1 g/L suspended in the reactor. The distance between UVA LED lamps and reactor was 15 cm and the light intensity measured was measured as 20 mW $cm^{-2}$. The representative of the six major groups of antibiotics, including polymyxin (PB), cefotaxime (CTX), azithromycin (AZI), tetracycline (TET), ciprofloxacin (CIP), and streptomycin (SM) (Sangon, China), were prepared from powder stocks and renewed before degradation, where CTX represents the most widely used β-lactam drug in decades and PB represents the most recently cracked super antibiotic [29]. The AZI, TET and CIP represent the most frequently determined and commonest antibiotics in clinical use. Besides, both of these antibiotics and corresponding ARGs were detected in environmental water system with very high frequency [30–33]. Five different concentrations including 16, 32, 64, 128, and 256 ppm were used for each antibiotic.

### 2.2. Determine the incubation period of bacteria during PC inactivation

To ensure that bacteria were still alive to actively respond to each stimulation, the time for sampling and analysis was controlled within the initial period called the incubation period, in which the curves of time-killing exhibit a “shoulder” and the value of $S_L$ (shoulder length) represents the time of incubation period. To determine the incubation period of each inactivation process, the Microsoft Excel tool called GInaFIT [34] was applied to produce curves fitting by a model of Log-linear + Shoulder, Log-linear + Tail and Log-linear + shoulder + Tail.

$$\frac{dCc}{dt} = -k_{max} \bullet C_c \quad (1)$$

$$\frac{dN}{dt} = -k_{max} \bullet N \bullet \left(\frac{1}{1 + C_c}\right) \bullet \left(1 - \frac{N_{res}}{N}\right) \quad (2)$$

The shoulder of the death curve was modelled by introducing the concept of a critical component ($C_e$, which may or may not be an actual substance within or outside the cell) that decays immediately upon ROS challenge. The amount of this component at the start of the challenge is a measure of the “physiological condition” of the cells and (together with the actual death rate) determines the length of the shoulder. Component $C$ decays as Eq. (1), where $C_c$ is related to the bacterial physiological condition [-], $k_{max}$ is the specific inactivation rate [1/time unit], and $N_{res}$ is the residual population density [cfu $mL^{-1}$]. The model uses four degrees of freedom: two initial states $N$ (0) represent the initial population of bacteria [cfu $mL^{-1}$] and $C_c$ (0) [-], and two parameter values $k_{max}$ and $N_{res}$.

### 2.3. Establishment of bacterial induction process mediated by PC stimulation

Considering that the bacterial response could be improved by PC stimulation, we further determined whether the bacterial antibiotic susceptibility would change with long-term PC stimulation. On the natural mineral interface, most of bacteria cannot be continuously stimulated by PC, due to their mobility. The PC stimulation might occur intermittently and transiently. Hence, two types of induction processes, PC induction and cross-induction, were designed. For PC induction, in the cycle of each day, after 15 min of PC stimulation, *E. coli* DH5α (CTX) was moved to LB nutrient for 15 min to avoid marked inactivation to ensure that the bacteria could grow during the whole process of induction and accumulate stress bacteria after cycles of induction. After 14 cycles, the bacteria were moved to LB and cultivated overnight (Fig. S1a). Notably, there was no antibiotic exposure during the entire PC-induction process. For cross-induction, two kinds of stimulation occurred alternately between PC and sublethal concentrations of antibiotics. That is, after 15 min of PC stimulation, *E. coli* DH5α (CTX) was moved to LB nutrient containing sublethal concentrations of antibiotics (PB, CTX, AZI, TET, CIP, SM) for overnight cultivation (Fig. S1b). As a control, before overnight cultivation in antibiotics containing LB nutrient, *E. coli* DH5α (CTX) were cultured in pure LB nutrient for 15 min without any other stimuli.

### 2.4. Assay the change of bacterial susceptibility to antibiotics after PC stimulation

For *E. coli* DH5α (CTX), MIC and inhibition zone for PB, CTX, AZI, TET, CIP, SM were measured every day of lethal-PC or lethal-PC and sublethal-antibiotic cross induction. Briefly, the $OD_{600}$ (absorbance value in 600 wavelength, maximum absorption wavelength of bacteria)

of the obtained bacteria from the process of induction was adjusted to 0.1 and then diluted 1:100 into 20 mL of Mueller-Hinton Broth (MH) nutrient. Approximately $10^6$ cfu $mL^{-1}$ of bacteria was subsequently moved to a 96-well plate containing different concentrations of antibiotics through doubling dilution and cultivated for 18 h at 37 °C. To determine the inhibition zone diameter, $10^6$ cfu $mL^{-1}$ of bacteria were spread evenly over the plate through swab before the antimicrobial susceptibility disk was placed on the surface of the plate and grown for 18 h at 37 °C.

### 2.5. Determine how bacteria stress response to the stimulation of PC

For RNA extraction, 1.5 mL of sample was collected from each treatment at regular intervals and centrifuged to remove reaction solution. Then, the total intracellular RNA was extracted using a Spin Column Bacterial Total RNA Purification Kit (Sangon, China) according to the manufacturer's protocol. RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription was performed using 5 μg of total RNA in a 10 μL reverse transcriptase reaction mixture using PrimeScript™ Master Mix (Perfect Real Time) (Takara, Japan) according to the manufacturer's instructions.

The expression levels of the stress response-related genes were determined using q-PCR. The *gapA* and *rrsA* genes were used as housekeeping genes because they did not exhibit any significant variation in expression among samples. Specific primer pairs (Table S1) were synthesized by Shanghai Sangon Biotech Co., Ltd. The detailed q-PCR procedure is described in the supplementary materials.

Up/down–regulation of gene expression is defined as a 2-fold change compared with that of the untreated sample in q-PCR. Origin9.0 was applied to draw the heatmap of gene expression.

### 2.6. Assay the occurrence of bacterial oxidative stress responses under the stimulation of PC

The probe 2′,7′- dichlorodihydrofluorescein diacetate (DCFH-DA) was adopted to monitor the intracellular ROS (including $H_2O_2$, $^{\bullet}OH$, $O_2^{\bullet -}$) levels [35]. The non-fluorescent probe DCFH-DA can freely penetrate into bacterial cells and be hydrolysed by intracellular esterase and further oxidized by intracellular ROSs to form fluorescent 2′,7′-dichlorofluorescein (DCF), which cannot penetrate into the cell membrane. Thus, the probe can be easily packaged in bacterial cells for fluorescent detection. During the assay, a regent, RSup, increased the concentration of ROSs in the cells was used as a positive control.

To measure the ability of maintaining redox equilibrium, which is represented by the activity changes of intracellular catalase (CAT, quenching $H_2O_2$) and superoxide dismutase (SOD, quenching $O_2^{\bullet -}$), bacteria treated in each process were sampled at regular intervals. The Bacterial Protein Extraction Kit (Sangon, China) was applied for total protein extraction, and the Modified Bradford Protein Assay Kit (Sangon, China) was used to measure the concentration of extracted protein before CAT and SOD activity assays. The procedures for CAT and SOD activity assays were performed with the Catalase Assay Kit and the Superoxide Dismutase Assay Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions.

### 2.7. Observation of morphological changes of bacteria after PC exposure by scanning electron microscopy (SEM)

During the treatment process, the bacterial cells were harvested and spread onto poly- L-lysine-coated slide glass directly and washed three times with phosphate buffer salt (PBS, pH 7.4) in a 1.5-mL EP tube. Then, the bacterial cells were prefixed in 2.5 % glutaraldehyde for approximately 2 h at 4 °C, dehydrated by a graded series of ethanol (30 % for 10 min, 50 % for 10 min, 70 % for 10 min, 90 % for 10 min and 100 % for 10 min 2 times). After fixation and dehydration, the slide was air dried in a drying vessel at room temperature overnight. Then, the sample was coated with gold before visualizing using a field emission SEM (ZEISS Ultra 55, Carl Zeiss, Germany).

### 2.8. Assay the effects of PC on express change of bacterial efflux pump by laser confocal microscopy characterization

For intracellular fluorescent antibiotic accumulation measurement, bacteria harvested from induction and normal growth were washed with PBS and moved to a 20-mL PBS solution containing 128 mg $L^{-1}$ of ofloxacin (OFX) (excitation/emission: 300 nm/507 nm). At 20, 40, 30 min, bacteria were coated onto poly-L-lysine-coated glass slides. The imaging of bacteria was performed on a laser confocal microscope (Carl Zesis LSM 800 with Airyscan, German) with a filter block N UV-A consisting of excitation filter Ex 300-375.

### 2.9. Determination of effects of PC on bacterial mutation rate

The mutation rates for each bacterial strain were examined following 18 h of growth in a 2 × MIC antibiotic-containing and plain LB plate. After colony counting from 10 replicates, the frequency of mutation was calculated by $C_0/C$, where $C_0$ (cfu of fitted mutation/mL) and C (cfu of viable bacteria/mL).

### 2.10. Statistical analysis

Analysis of variance (ANOVA) was used to determine significant differences in the gene expression of antibiotic-tolerant strains. Relative ROS, anti-enzyme activities, and cell density calculations were replicated three times, and the results are expressed as the means ± standard deviations (n = 2).

## 3. Results

### 3.1. Bacterial stress response under exposure to PC, CTX and PB

The stress response, as an important reaction of bacterial antibiotic resistance, can play a vital role in the process of antibiotic exposure [36]. In this study, before investigating the influence of PC on the development of bacterial antibiotic resistance, we first determined how bacteria stress response to PC stimulation. To ensure that the results can precisely reflect the bacterial responses, bacterial cells must be viable during the period of stress state analysis. Here, through GlnaFit [34] curve fitting, the time-killing curves were plotted to determine the incubation period (Table S2) by which bacteria could survive before massive death. The cell integrity was also verified during the calculated incubation time through SEM (Fig. S2). From the obtained data and adjustment intensity of the process of each treatment, the initial 30 min was defined as the incubation period in this study, and subsequent mechanism studies were conducted within the incubation period. To explore how the bacterial stress response under PC stimulation, q-PCR was applied to analyse the alternation of stress gene expression patterns of four constructed strains of *E. coli*, including one antibiotic-susceptible strain (*E. coli* DH5α), two strains containing single-ARG (antibiotic-resistant gene) (*E. coli* DH5α (CTX) and *E. coli* DH5α (PB)), and one strain containing double-ARG (*E. coli* DH5α (CTX, PB)) (Materials and Methods). The selected stress response-related genes include nutrient/growth impairment, oxidative stress, envelope stress, and SOS stress, all of which are also relevant to bacterial inherent antibiotic resistance, and the detailed information is listed in Table S3.

As shown in Fig. 1a and b, when these bacterial strains were exposed to antibiotics CTX or PB, the relative expression level of some stress genes of the four test strains increased more than 2-fold. The increase of gene expression level is defined as up-regulated (enhanced in gene expression) in this study. The red colour of this gene expression area suggested that bacteria arouse selected stress responses with the

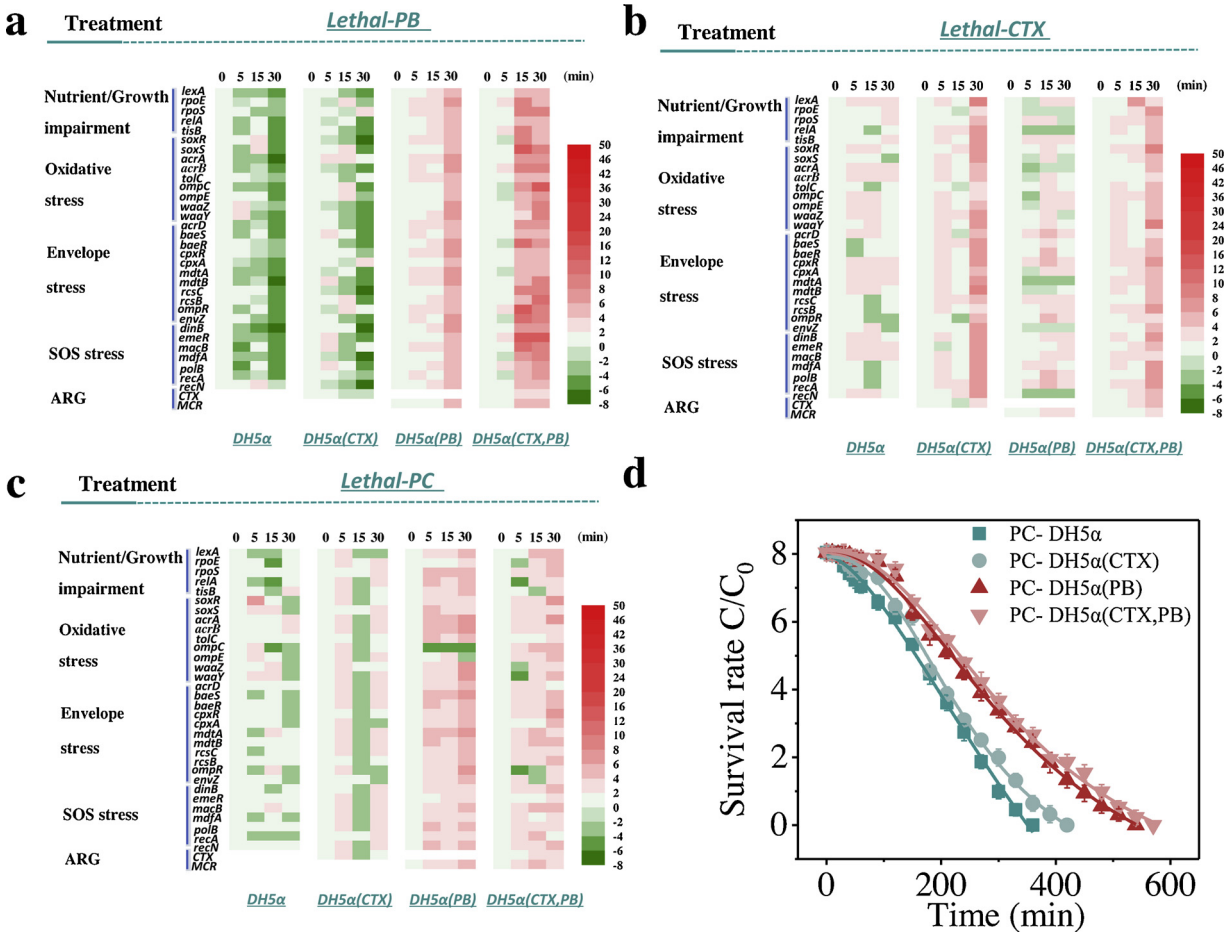


**Fig. 1.** PC could induce bacterial stress responses that related to antibiotic resistance. Temporal genes relative expression value profiles of *E. coli* DH5α, DH5α (PB), DH5α (CTX), DH5α (PB, CTX) elicited by (a) lethal-PB (b) lethal-CTX (**c**) lethal-PC during the incubation period by cluster analysis according to bacterial stress response. Each column represents a time point. Each row represents one gene. The shade of color represents change in relative expression value: red, up-regulation; white, no change; green, down-regulation. The profiles were divided into 5 clusters: Nutrient/growth impairment; Oxidative stress; Envelope stress; SOS stress; ARG. (d) Time killing-curves of *E. coli* DH5α, DH5α (PB), DH5α (CTX), DH5α (PB, CTX) through the lethal-PC stimulation.

up-regulated expression of stress genes that confer protection. It is reasonable that bacteria could positively respond to these antibiotics through various stress response systems because the selected stress genes are also related to some antibiotic resistance mechanisms, such as efflux pumps, lipopolysaccharide modifications and decreased cell permeability, which specifically play an important role in antibiotic resistance. However, interestingly, the expression of the selected genes was also up-regulated under the stimulation of lethal-PC (Fig. 1c), indicating that in addition to the tested antibiotics, these antibiotic-related stress responses can also be elicited by PC stimulation. Recent studies have shown that many bacterial stress responses could be linked to bacterial antibiotic resistance. For example, oxidative stress could regulate the expression of *soxSR-waaZY*, which mediate the bacterial modification of lipopolysaccharide and, therefore, endow a certain degree of antibiotic resistance [37]. Mutations with the overexpression (stronger expression activity) of some stress genes, such as efflux pump genes *cpxRA* or *BaeSR*, could alter their bacterial susceptibility to specific antibiotics [12]. In addition, the genes related to the bacterial phenotype of biofilm and the occurrence of persistent bacteria, such as *rpoS* (encoding protein factors that regulate bacterial stress) and *lexA-tisAB* (regulating the formation of biofilm and the occurrence of persistent bacterium), also exhibit the inherent or improved tolerance to antibiotics [38]. In this study, during the PC process, these genes were all up-regulated, which suggests an association of PC stimulation with bacterial antibiotic susceptibility. Individual stresses could activate bacterial general stress responses and coexist with other specific pathways to survive under various stresses [39,40]. For example, starvation induction could cross protect against heat or $H_2O_2$ challenge in *E. coli* [41]. Herewith in our study, the bacterial stress responses under PC stimulation might also come from general bacterial stress, by which bacteria could mediate various specific stress response mechanisms to help their survival through cross-protection when they face other individual stresses [40].

Compared with the non-ARG-containing *E. coli* strain, higher intensive stress responses together with a higher degree of the up-regulated expression of stress response-related genes and ARG were found in the ARG-containing strains that encounter either antibiotics or PC stimulation. The ARG could not only favour the resistance to antibiotics specifically but also work to respond to stimuli other than antibiotics and even enhance other bacterial stress responses. Consistently, ARG-containing bacteria have a longer incubation period and persist longer during the entire process of PC stimulus, indicating that ARG-containing bacteria gain higher tolerance to PC than antibiotic-susceptible bacteria (Fig. 1d). It seems that the ARG serves as an 'enhancement gene' to improve bacterial survival under other stimuli other than antibiotics and belongs to the bacterial inherent stress system so that participates in cross-protection. Reverse thinking, it is reasonable, for example, that *soxRS,* the bacterial inherent stress response gene that responds to oxidative stress, could also mediate the expression of the efflux pump gene *acrAB-tolC* [11,42] and the lipopolysaccharide modification gene *waaYZ* [37] to play an important role in bacterial antibiotic resistance, where we can also define *soxR* as an "enhancement

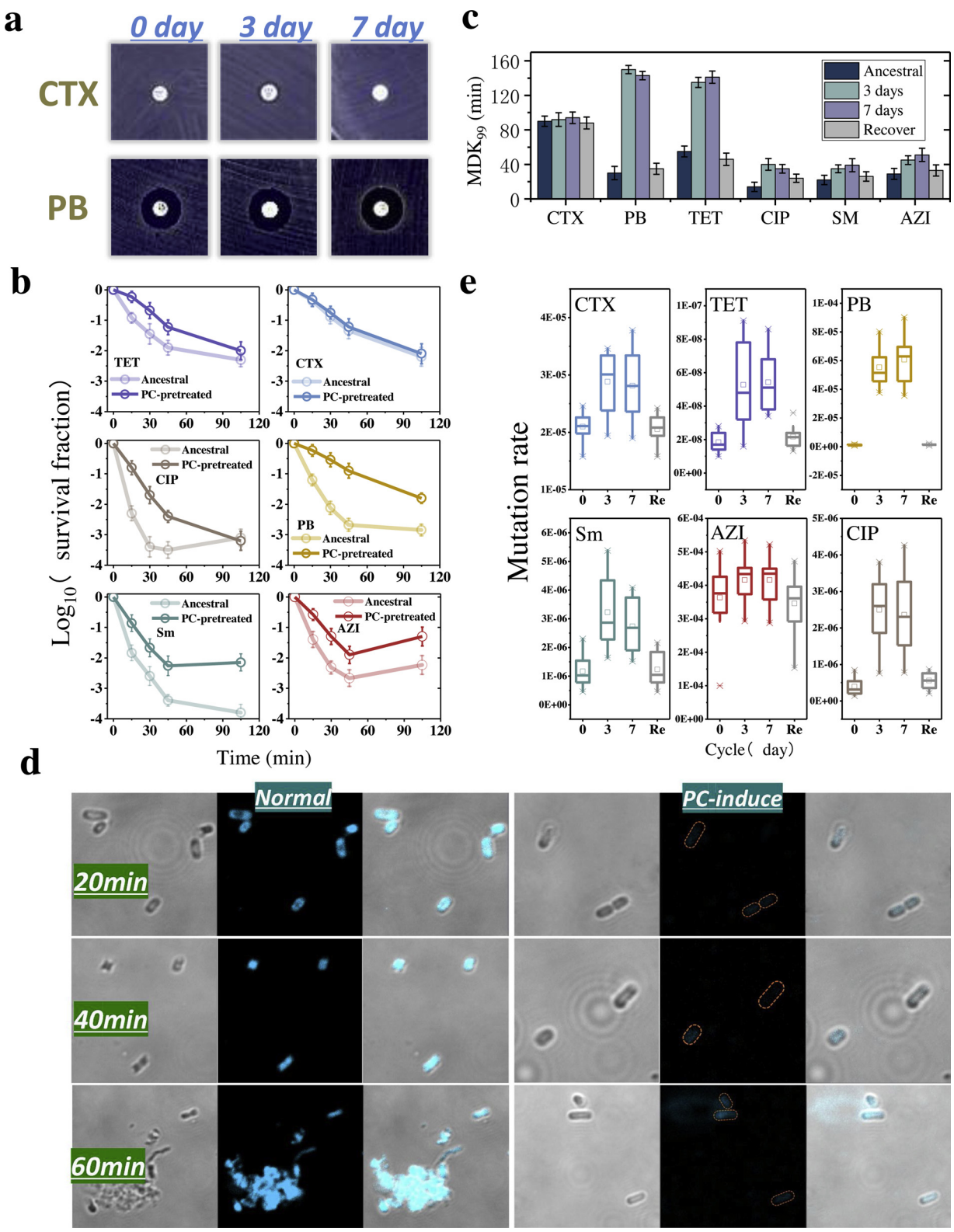


**Fig. 2.** Improved bacterial antibiotic-tolerance after induction of lethal-PC. (a) Inhibition zone assay of PB and CTX after 3 and 7 days of induction. (b) Time-killing curves of DH5α (CTX) of different class of antibiotics after PC pretreated or not. Data are presented as the mean ± s.d. of three independent experiments (**c**) $MDK_{99}$ was determined by measuring the time to kill 99 % of the population. Data are presented as the mean ± s.d. of three independent experiments (d) Fluorescence microscope observed the accumulation of Ofloxacin (OFX) in bacterial intracellular based on fluorescence of OFX (maximum excitation wave length = 290 nm; Emission wave length = 507 nm) (**e**) The mutation rates of DH5α (CTX) faced to each kind of antibiotic (2 × MIC) after t days (where t = 0, 3, 7) of lethal-PC induction; Re represented the bacteria that recovered from induction and growth in LB nutrient one more day.

gene". As such, there may be some other side functions of ARG, which are equal to other stress response genes in some perspective, and these stress-related genes and ARG might belong to a defence system and work together.

Usually, when bacteria undergo threatening conditions, such as antibiotics or PC oxidation, the bacteria will lose vitality and show an intracellular imbalance of redox state [43,44]. Hence, the bacterial oxidative stress responses were further investigated at the protein level in this study. That is, two main anti-oxidative enzymes, CAT and SOD, were selected to determine the variation of enzyme activities during PC and antibiotic stimulation, respectively. In addition, the level of intracellular ROS was also analysed to evaluate the oxidative stress of bacteria. As shown in Fig. S3, intracellular ROS levels increased within 30 min of incubation for all tested treatments, particularly under PC stimulation, which might be contributed by the penetration of ROS, generated by PC, from extracellular into intracellular of bacterial cells. Besides, the damage effects of PC could disturb normal metabolism pathway, which might destroy the redox equilibrium in bacterial intracellular, leading to the increased level of ROS [27]. In each process, the intracellular ROS level in the tested antibiotic-susceptible bacterial strain obviously increased in the presence of antibiotics (CTX or PB) or PC stimulation. This result is consistent with the hypothesis that the antibiotics could induce bacteria death by destroying its antioxidant system, leading the increase of ROS level [45–47]. Comparatively, ARG-containing bacteria showed a smaller variation range, which corresponded with the results showing that the ARG-containing bacteria were more tolerant to each stimulus and maintained a better redox equilibrium (Fig. S3). This smaller variation of intracellular ROSs might be contributed by higher expression of anti-oxidation enzymes through the oxidative stress response [48,49]. However, the changes in anti-oxidation enzyme activities were still controversial. That is, the enzyme activities of SOD and CAT in ARG-containing bacteria only changed slightly during the incubation period as compared with those in the antibiotic-susceptible bacterial strain (Figs. S4 and S5), which might be ascribed to higher intensity of other stress responses through cross-protection [39,40] but not specific oxidative stress.

### 3.2. Increased intensity of stress responses under exposure of joint stimulus

To take the actual complex environment into consideration, the stress responses under a joint stimulus system were further investigated. To simplify the system, the stimulation included PC plus antibiotics (schematic diagram of PC plus antibiotic process is shown in Fig. S6), two kinds of antibiotics and PC plus two kinds of antibiotics. When multiple stimuli (PC + antibiotics) were applied together, it could be seen that the colour of gene express areas were exhibited redder than that in single stimulus process (Fig. S7), meaning that the stress responses of related genes were more up-regulated expressed under the combination system. This result indicated that the intensity of stress responses could be enhanced when multiple stimuli were applied together. However, even with enhanced stress responses, the ROS level showed a higher increase during the joint system than that in the single stimulus (Fig. S3), indicating that the joint system posed more serious stimulation and the bacterial redox state was greatly impacted. Correspondingly, the anti-oxidative enzyme activities of SOD and CAT increased slightly to facilitate survival (Figs. S4 and S5). Based on the results in the joint system, it could be deduced that in the actual environment, with the addition of stimulus, bacteria could become more sophisticated and arouse more intensive stress responses to become more tolerant to the aggression of external factors, such as antibiotics. Therefore, we hypothesized that this improvement of stress responses could originate from other environmental stimulations, even without the existence of antibiotics.

### 3.3. PC induction improved bacterial antibiotic tolerance

It has been proposed that the general stress response with coexisting specific stress responses, such as oxidative stress and SOS stress, enables bacteria to cross-protect by anticipating stressors that may not be present at the moment but are likely to occur soon [39]. For example, pre-exposure to a specific stressor enhances bacterial resistance to different stressors applied subsequently [41,50]. Thus, we expect that short-term PC stimulation might also improve bacterial antibiotic tolerance, and long-term PC induction might alter bacterial susceptibility to some antibiotics. Therefore, to investigate bacterial variation of antibiotic susceptibility through intermittent lethal-PC induction (the detailed induction procedure shown in Materials and Methods), the single-ARG containing *E. coli* DH5α (CTX) was chosen as the model antibiotic-resistant bacteria to investigate the influence of the induction process on bacterial antibiotic susceptibility. Six different classes of antibiotics (PB, CTX, AZI, TET, CIP, and SM) (Table S4) were chosen to measure their MICs. Unexpectedly, the results of MICs were not changed for all six antibiotics during 30 days of induction (Table S5), and the change in the inhibition zone diameter was indistinguishable after 3- and 7-day induction (Figs. 2a and S8), indicating that bacterial susceptibility to antibiotics did not change. This result was consistent with some other study, where it is been reported that the bacterial antibiotic resistance to TET and VAN cannot significant changed by PC in Urban wastewater [24]. However, different from this study, some work reported that the PC inactivation could reduce bacterial resistance to tetracycline [23]. Besides, it is also been reported that the antibiotic resistance of bacterial could be altered, where some bacterial species increase its susceptibilities while some other bacterial species decreased instead [22]. On the other hand, under the lethal-PC stimulation, the bacteria might become antibiotic-tolerant but not antibiotic-fitted. Unlike the sublethal dose of induction [51], the growth of the bacteria might be suppressed, and the antibiotic-fitted strain would not be accumulated during lethal-PC stimulation, posing an obstacle for the development of bacterial antibiotic resistance. Similar to this study, even if the lethal factor was driven by a high lethal concentration of antibiotics, bacteria cannot evolve to become antibiotic-resistant bacterial strains directly, and no change in susceptibility to antibiotics was found [52].

Although the stress responses cannot change bacterial resistance to antibiotics in this study, as we expected, the bacterial antibiotic tolerance does obviously change during the PC stimulation. As shown in Fig. 2b, after only one cycle of PC induction, the bacterial incubation period in the antibiotic killing process was prolonged. The $MDK_{99}$ (the time for killing 99 % of cells) [52] increased from 30 to 150 min for PB, 55–135 min for TET, 14–40 min for CIP, 22–35 min for SM, and 29–45 min for AZI after the first cycle of induction and was sustained with further induction (Fig. 2c). These indicate that *E. coli* DH5α (CTX) become more tolerant to antibiotics. For $MDK_{99}$ of CTX, no obvious change was observed. This effect is probably because the bacterium used in this section was originally a CTX-resistant strain. Notably, these results were consistent with the up-regulated expression of genes related to efflux pump and permeability reduction, which belong to envelope and oxidative stress responses, as shown in Figs. 1a–c and S7, indicating that bacteria might show less intracellular accumulation of antibiotics to help their survival. This hypothesis was further confirmed by laser confocal fluorescence microscope characterization, as shown in Fig. 2d. After 20, 40, and 60 min of exposure to OFX, the fluorescence intensity of untreated *E. coli* DH5α (CTX) obviously increased due to the accumulation of OFX in the cell. In contrast, after PC induction, bacteria showed lower fluorescence intensity than untreated bacteria at the corresponding sample time, suggesting that lower accumulation of antibiotics was found in the PC-induced bacterial cells; if they were subsequently exposed to antibiotics, their antibiotic tolerance would be improved.

Furthermore, SEM was employed to examine the performance of *E. coli* DH5α (CTX) during the cross-induction process. As shown in Fig.

S9, the individual bacterial cells were stuck to each other, which exhibited a biofilm phenotype. Biofilm is one of the pathways of the stress response, and it has higher antibiotic tolerance than the corresponding planktonic cells [53]. Therefore, our results indicated that some of the planktonic bacterial cells switched to a biofilm (slide glass substrate) mode of growth in response to the above-mentioned PC induction.

Basically, when bacteria face a threatening condition, their mutation rate will increase through the mechanism of stress-induced mutagenesis to facilitate the occurrence of fitted variants [54]. Herewith, the mutation rates of *E. coli* DH5α (CTX) also increased approximately 3–5 times to the six kinds of antibiotics tested after lethal-PC induction (Fig. 2e), suggesting that more fitted variants might be produced under the selection of antibiotics, which might accelerate the development of antibiotic-resistance strains in specific bacterial communities. However, further study found that when the bacteria were re-cultured in LB nutrient after 7-day induction, the improved antibiotic tolerance of the produced mutants disappeared, and the $MDK_{99}$ returned to the original values (Fig. 2c). In addition, the mutation rates also returned to normal levels when PC stimulation was removed (Fig. 2e). These results indicate that the improved antibiotic tolerance contributed by stress responses was temporary and reversible when under the appropriate growth conditions.

A previous study reported that in addition to antibiotics, some treatments, such as $H_2O_2$, could also induce bacterial-specific mutations to contribute bacterial antibiotic resistance directly through the overexpression of a specific stress response gene [55]. Therefore, to investigate whether the antibiotic-fitted variants could directly attribute to the lethal-PC induction through the overexpression of stress response genes, the gene expression of randomly selected variants from antibiotic-containing plates was also examined. Unexpectedly, no obvious stress responses were aroused with less than a 2-fold change in stress gene expression (Fig. 3), and the antibiotic-sensitive spectrum of variants depended only on the type of screening antibiotics (Table S6). These results were in good agreement with our above-obtained phenomenon of reversible antibiotic tolerance. That is, unlike genetic-mediated antibiotic resistance, the stress-related antibiotic tolerance was nongenetic and nonheritable. Overall, a conclusion can be tentatively drawn that lethal-PC induction could transiently improve bacterial nonheritable antibiotic tolerance.

### *3.4. Cross-induction facilitates the evolution of bacterial antibiotic resistance*

Previous studies have found that the gradual adaption of bacteria to antibiotics might result from constant selection of many antibiotic-fitted mutations during exposure to sublethal concentration of antibiotics [5]. However, in this study, even though lethal-PC stimulation could improve bacterial antibiotic tolerance, no selection of antibiotics or accumulation of specific antibiotic-fitted mutations were found, as mentioned above. Nevertheless, there is a possibility that antibiotic-tolerant bacteria can boost the chances for antibiotic-resistant mutations and pave the way for the rapid subsequent evolution of bacterial antibiotic resistance [56]. Hence, we hypothesize that bacterial stress responses caused by PC induction could transiently improve their antibiotic-tolerance, and these antibiotic-tolerance bacteria could evolve to antibiotic-resistance faster through the subsequent selection pressure of sublethal antibiotic concentrations. To confirm this hypothesis, the cross-induction experiments were further designed to reveal how lethal PC stimulation contributed to the development of bacterial antibiotic resistance. After cross-induction each day, the MIC of six antibiotics was measured. As shown in Fig. 4, after 7-day cross-induction without intermittent stimulation of PC (control), the fold change of MIC only reached 14 for SM, 8 for PB, 8 for CTX, 2 for AZI, 8 for CIP, and 8 for TET. Comparatively, after 7-day cross-induction, the fold change of MIC was up to 16 for SM, 32 for PB, 16 for CTX, 4 for AZI, 16 for CIP, and 32 for TET, which are much higher than those of normal induction. This finding indicated that the evolution of bacterial antibiotic resistance was accelerated through cross-induction, which coincided with our hypothesis. That is, the temporarily improved tolerance by stress responses do facilitate the development of bacterial antibiotic resistance when the bacteria are subjected to the selection pressure of sublethal concentrations of antibiotics.

Furthermore, to understand whether any other lethal factors could also contribute to the development of antibiotic resistance, the lethal-TET or sublethal-TET was used to replace lethal-PC during the stimulation process of cross-induction. As expected, pre-treatment with lethal-TET also facilitated the development of SM resistance (Fig. S10). In contrast, after pre-treatment with sublethal-TET, the bacteria did not develop SM resistance (Fig. S10). It might be explained that, at the sublethal concentration of antibiotics, the selection pressure was dominant during the whole process, which might even suppress the development of next antibiotic resistance due to fitness cost [57].

## 4. Discussion

The main goal of this work is to investigate how PC affects bacterial stress responses and the development of bacterial antibiotic resistance. We found that lethal-PC stimulation could elicit bacterial stress responses to improve antibiotic tolerance during the incubation period, which could further accelerate the development of antibiotic resistance under subsequent exposure to sublethal concentration of antibiotics but not directly contribute to antibiotic resistance. Several previous studies have mainly focused on the contribution of other anthropogenic or environmental factors, except some sublethal concentration of components, which might have some similarities to antibiotics, to the occurrence of bacterial antibiotic resistance directly through selection pressure [58]. Different from their results, in this study, bacteria cannot evolve to antibiotic-resistant during the lethal-PC induction. However, with different PC system, some other study reported that the antibiotic resistance of some bacterial strain could be altered with increase or decrease to different antibiotics [22,23]. Besides, after PC inactivation, the alternation of bacterial antibiotic resistance shows in different ways to different type of antibiotics with slightly increase or not significant change [59]. It seems complicated that the change of bacteria antibiotic resistance was counted on many factors such as different PC system, different bacterial species and different antibiotics used. Thus, it is hard to conclude that the PC possesses the ability to alter the antibiotic susceptibility of bacteria or not.

Although bacteria cannot transfer from antibiotic-susceptible to antibiotic-resistance after the induction of lethal-PC, we do suggest that there may be some relationships between PC stimulation and bacterial antibiotic-tolerance development according to the results of stress responses in this study, where PC could also elicit a similar bacterial stress response system as antibiotics. Bacteria arouse a set of stress responses to survival and by the way to improve their antibiotic tolerance, which make bacterium more endurable under temporary antibiotic treatment.

Additionally, under lethal-PC stimulation, all the tested stress responses were aroused through different mechanisms, such as efflux pump, stress-induced mutagenesis, biofilm formation, DNA repair or modification, to improve bacterial antibiotic tolerance. The various stress responses might be caused by the accumulation of global regulators sigma S (protein factors that regulate bacterial stress), which could stimulates various kinds of stress to work together [60]. Given these results, we suggested that bacteria could arouse generally and their coexisted specific stress responses under lethal-PC stimulation, which could be termed “chastening experience”, to train and improve bacterial abilities to survive under changing environments. In addition, stress induction could also facilitate bacterial mutations and accelerate bacterial fitness under various environmental conditions. As the saying goes, “men must choose between progress and comfort”, and the “chastening experience” could facilitate bacterial antibiotic tolerance in a hostile environment. However, this improved tolerance through the

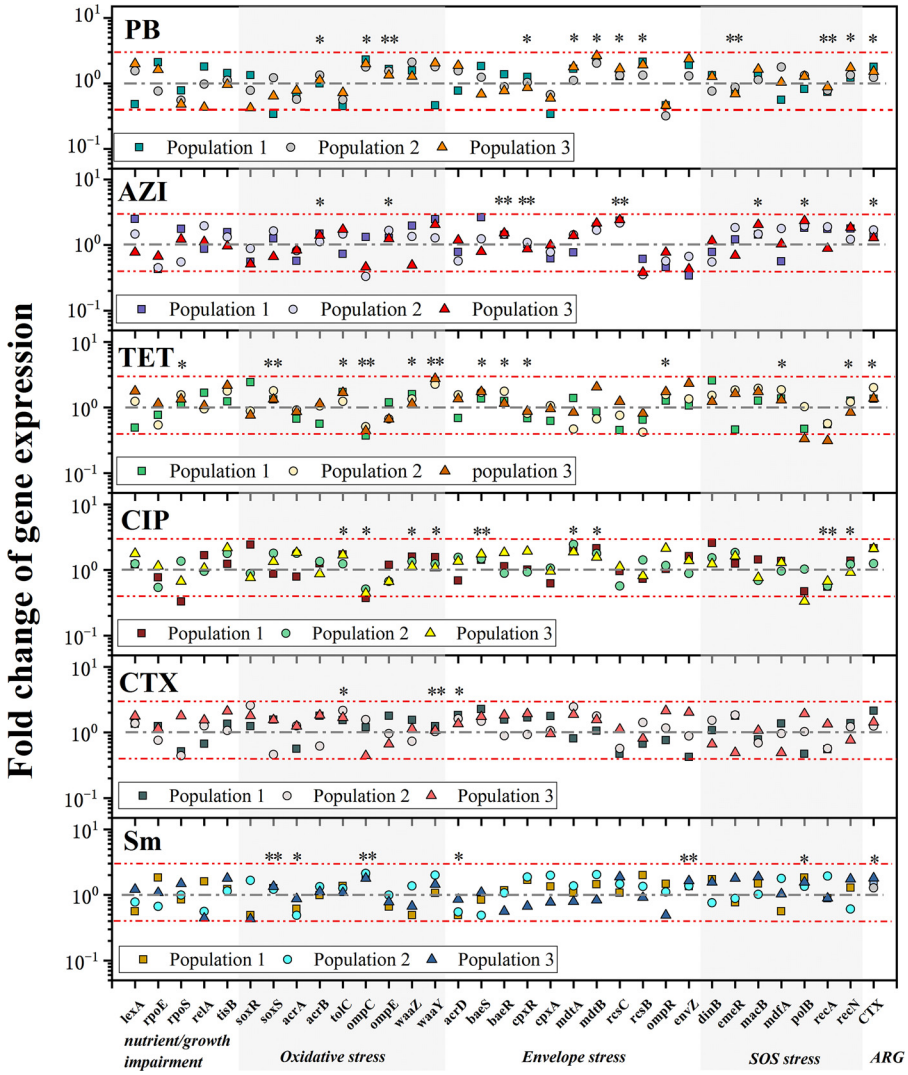


**Fig. 3.** The stress responses related gene expression of randomly selected mutants from different antibiotics after PC induction. Red dashed line represented 2 folds change (up or down regulated) of gene expression. Up/Down–regulation of genes expression was defined as more than 2-fold change compared with that of the untreated sample. Analysis of variance (ANOVA) were used to determine significant differences in bacterial gene expression. Significant differential expression is denoted with $*$ for $P < 0.05$ and $**$ for $P < 0.01$.

stress responses is reversible and not inheritable, which cannot be attributable to genetic changes [61]. Although this kind of tolerance may not immediately contribute to the occurrence of bacterial antibiotic resistance, it did set the stage for the development of more efficient resistance factors with enabling bacteria to withstand low-level antibiotic exposure for longer times or reduced fitness cost (The bacterial growth cost of adapting to a new environment) [62]. Consistently, the speed of bacterial evolution to antibiotic resistance under sublethal concentration of antibiotics could be first accelerated through the cross-induction with lethal-PC stimulation in each cycle in this study, indicating that stress improved bacterial antibiotic tolerance and then accelerated the occurrence and accumulation of antibiotic-fitted mutations. In agreement with these results, antibiotic-tolerant persistence bacteria can usually boost the chance for antibiotic-resistance mutations and pave the way for the rapid subsequent evolution of resistance [56]. Furthermore, except for PC, some other environmental factors, such as heat or starvation, may also cross-protect (several protection mechanisms can be in common to each other) bacteria and confer bacterial antibiotic resistance to other stresses [41,50]. Thus, considering the environmental process, the development of bacterial antibiotic resistance might also be facilitated by different bacterial "chastening experience", such as heavy metals, toxicants, biocides and some other unfriendly components or unbalanced conditions, such as PC oxidation technology in this study. Therefore, the bacterial antibiotic resistance was not an independent consequence of not only antibiotics but also the whole environmental system. That is, during the evolution process of bacterial antibiotic resistance, the unique function of antibiotics only posed a specific selection pressure, while many other threatening environmental conditions produced naturally or anthropogenically could also speed up the development of bacterial antibiotic resistance. The overuse of antibiotics in the clinic or through some other pathways was only one of the reasons for bacterial antibiotic resistance, but the fundamental reason might be the occurrence of unbalanced environmental threatening conditions or anthropogenic disturbance in the ecosystem. The existence of residual antibiotics is also a problem of unbalanced environmental conditions, to say the least.

Furthermore, we found an additive effect on bacterial stress responses under multi-stimuli that elicits a more intensive protective mechanism; the more bacteria will chasten, the higher the intensity of stress responses will be elicited. Hence, we suggest that the worse the environmental condition is, the more bacteria will chasten, and the more seriously the problem of antibiotic resistance will emerge. In addition, the ARG works to some extent as an "enhancement gene"

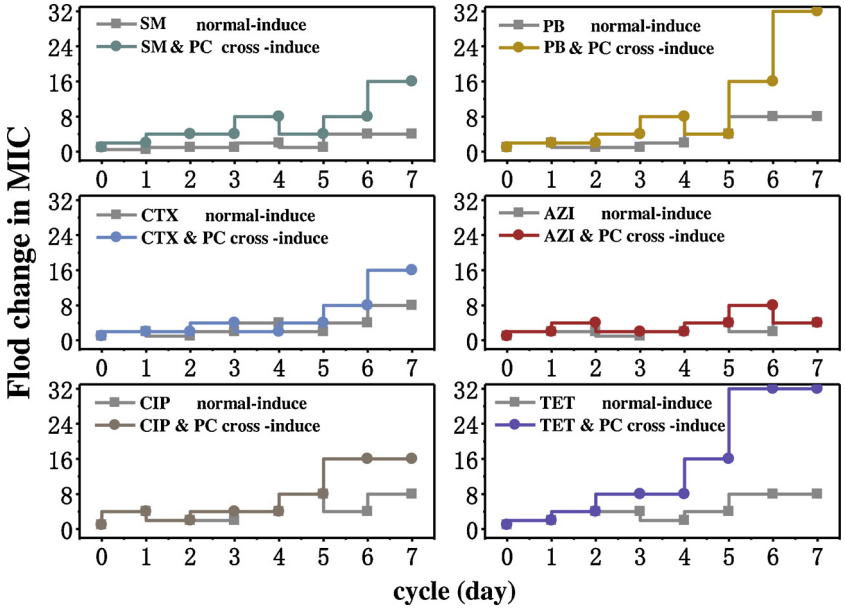


**Fig. 4.** Development of bacterial antibiotic-resistance was accelerated under lethal-PC Cross-induction. The alternation of bacterial antibiotic resistance was represented by the fold change of MIC to different antibiotics.

responds not only to antibiotics specifically but also to PC conditions and additively facilitates inherent bacterial stress responses.

## 5. Conclusions

Taken together, herein, we reported that PC oxidation technology as an adverse environmental or anthropogenic factor could contribute to the rapid development of bacterial antibiotic resistance through transient improvement of their antibiotic tolerance mediated by the stress responses first. By analogy, the natural environment usually works as a training ground for bacteria, which might also facilitate or magnify the problem of the antibiotic-resistance of bacteria. Therefore, increased knowledge of the anthropogenic or environmental factors that drive resistance may ultimately allow us to clarify how resistance emerges and its disseminated to develop more efficient mitigation strategies.

## CRediT authorship contribution statement

**Hongliang Yin:** Methodology, Writing - original draft. **Guiying Li:** Validation. **Xiaofang Chen:** Data curation. **Wanjun Wang:** Writing - review & editing. **Po Keung Wong:** Supervision. **Huijun Zhao:** Supervision. **Taicheng An:** Conceptualization, Supervision.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.apcatb.2020.118829.

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