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Regulatory formation of VBNC state antibiotic-resistant bacteria in water induced by sub-lethal photocatalysis and their resuscitation mechanism

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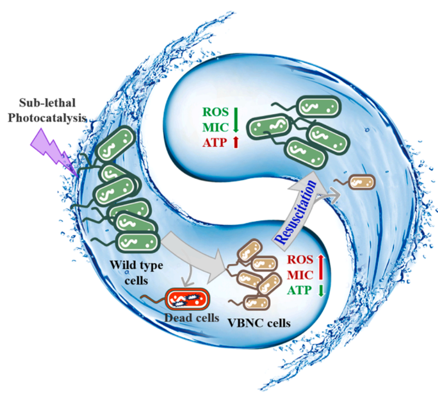
HIGHLIGHTS

- *E. coli* could be induced into VBNC state by sub-lethal photocatalysis.
- After removing stress, VBNC *E. coli* could resuscitate with recovered bacterial length, culturability and growth.
- Repairing damage caused by sub-lethal photocatalysis is critical for resuscitation.
- The major force driving resuscitation is intracellular ATP and metabolic activity.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of antibiotic-resistant bacteria (ARB) in water poses serious threat to public safety. Worse still, to escape damage, ARB could enter viable but nonculturable (VBNC) state during water disinfection, and VBNC bacteria may resuscitate and further exposure to human, even causing serious disease. Herein, both regulatory formation mechanisms of VBNC bacteria induced by sub-lethal photocatalysis and its resuscitation mechanism were elucidated. After inducing for 1 h, $> 10^8$ CFU/mL *E. coli* of all strains including ARB entered VBNC state, demonstrated by losing culturability and decreasing cell length. After stress rescinded for 5–10 d, VBNC *E. coli* could resuscitate, with a gradually increased trendy within 20 d and then decreased slightly. This resuscitation was demonstrated by recovering cell length, culturability and growth to the extent as wild type state. However, the resuscitated cells showed a higher antibiotic resistance than wild type cells. Furthermore, qPCR analysis indicates that the repaired oxidative damage and the induced changes in energy allocation were the driving force for resuscitation of VBNC *E. coli*. This study deepens our understanding of health risks associated with ARB

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entering and exiting VBNC state, and provides new insights into developing more effective strategies to water disinfection.

1. Introduction

Microbial pollution in water is a great global environmental and health safety issue. Therefore, effective water disinfection is great important to quality of water supply and human health [1]. However, microbes have various strategies to survive based on their living environment. During water disinfection, some bacteria will be killed directly, while some are still alive in active or dormant states. Among the dormant states, viable but nonculturable (VBNC) state is a representative dormant subpopulation. Entering VBNC state is a key strategy for bacteria to improve their survivability under harsh condition [2,3]. Unfortunately, water disinfection is also unfavorable stress for bacteria. Some water disinfection techniques, which intent to eradicate waterborne pathogens, can induce bacteria into VBNC state [4,5]. For instance, *Pseudomonas aeruginosa* and *Escherichia coli* are induced into VBNC state under ultraviolet radiation [6]. *Legionella* is induced into VBNC state by chlorine and chloramine [7,8]. VBNC *E. coli* is also detected during ozonation in municipal wastewater treatment plant [9]. Moreover, VBNC bacteria cannot be fully eliminated by these disinfection methods owing to their strong tolerance to various stress [4].

Worse still, water disinfection efficiency would also be overestimated because VBNC microorganisms cannot be detected using heterotrophic plate count method, due to the VBNC cells losing their culturability completely. Further, in VBNC state, some opportunistic pathogens still retain certain metabolic activity and virulence, posing a great threat to human health [10] and also closely associated with spread of antibiotic resistance and recurrent infection, resulting in longer hospital stays [11,12]. It is because, under favorable conditions or adversity being eliminated, the VBNC pathogens will resuscitate and regain high virulence as normal bacteria, imposing disease stress again [13]. That is, the survived VBNC bacteria during water disinfection may resuscitate when transportation in pipeline network [14]. To date, most previous studies have mainly focused on the resuscitation conditions, including temperature [15], nutrients [16], resuscitation promoting factors [17], and host cells [12]. What remains largely unexplored is whether disinfection-induced VBNC bacteria can resuscitate [18,19]. Even more striking, the underlying molecular mechanisms governing the resuscitation of the disinfection-induced VBNC cells have not yet been systematically elucidated.

Herein, we aimed to investigate the formation mechanism of VBNC bacteria induced by sub-lethal photocatalysis and their resuscitation mechanisms to normal state. To quantify the physiological and biochemical characteristics of the VBNC and resuscitated bacteria, the morphology, metabolic activity, and gene expression were measured using scanning electron microscopy (SEM), Laser Confocal Raman Microspectroscopy, and quantitative real time polymerase chain reaction (qPCR), respectively. Furthermore, the antibiotic resistance-related risks of resuscitated bacteria were assessed. Overall, this work will help us to clarify specific regulatory mechanisms of the resuscitation of VBNC bacteria.

2. Materials and methods

2.1. Bacterial strains and induction of VBNC bacteria

Antibiotic-resistant bacteria (ARB) (*E. coli* DH5α (MCR) and *E. coli* DH5α (CTX) constructed by us [20]) and antibiotic-susceptible bacteria (ASB) *E. coli* DH5α (Sangon, China) (Table S1) were investigated. Preparation of bacteria is provided in Supporting information (SI). The VBNC bacteria were induced in 50-mL quartz flask reactor with TiO_2 nanotubes illuminated with a 365 nm LED lamp (100 mW/cm^2) (Fig. S1) following the protocols from our previous article [20]. Under these conditions, a pure VBNC bacterial solution can be obtained without the presence of photocatalyst particles. Bacteria were sampled at different intervals. Control experiments (blank, ARB & ASB treated with TiO_2 nanotube or $UV_{365\,nm}$ alone) were also performed (Fig. S2). During the sub-lethal photocatalysis, the culturable cells were counted using heterotrophic plate count method. Total viable bacterial cells were identified with flow cytometry (BD FACSAria III, BD Biosciences) and LIVE/DEAD BacLight Bacterial Viability Kit (L7012, Molecular Probes, Inc., Eugene, USA). Detailed procedures are descripted in SI. VBNC cell numbers were calculated as the difference between viable and culturable cell numbers.

2.2. Resuscitation experiments, measurement of colony size and growth curves

After eliminating sub-lethal photocatalysis and standing for 5–60 d at room temperature, the colony formation was studied using plate paint method (cultured for 24 h at 37 ℃). Resuscitation occurred if culturable bacteria were observed. Number of resuscitated cells was counted and colonies were photographed. Agar surface covered by colony was calculated by analyzing photographs using ImageJ and used as a proxy for colony size.

Growth curves of bacteria before entering VBNC state (named as wild type (WT)) and the resuscitated cells were detected using LogPhase 600 Microplate reader (The LogPhase™ 600, BioTek, USA) as described in SI.

2.3. Characterization of VBNC and resuscitated cells

2.3.1. General characterization

Morphology of VBNC and resuscitated cells was observed with SEM. Intracellular reactive oxygen species (ROS) content was determined using Reactive Oxygen Species Assay Kit (Beyotime, China). Bacterial membrane permeability was determined using N-phenyl-1-naphthylamine [21]. Cellular ATP was determined using Enhanced ATP Assay Kit (Beyotime, China). Bacterial activity was determined using D_2O labeled Raman spectroscopy [22]. All detail information is provided in SI.

2.3.2. Detection of antibiotic resistance

The minimal inhibitory concentration (MIC) usually indicates the bacterial antibiotic resistance. However, MIC of broth micro-dilution method (BMD-MIC) is defined as the lowest concentration of antibiotics which resulted in complete inhibition of visible bacterial growth [11]. Because VBNC cells lost their culturability completely, their antibiotic resistance was detected as described below instead of abovementioned methods.

First, the MIC of WT cells was determined by broth microdilution as described in SI. Then, based on MIC values obtained, we set up a series of concentrations of respective antibiotics for all strains to determine antibiotic resistance. Prepared culturable, VBNC and resuscitated cells were added to the wells containing antibiotics (cefotaxime/polymyxin) incubated at 37 °C, and the antibiotic concentrations are listed in Table S2. Then, these cells were washed twice and resuspended in sterile 0.9 % NaCl. Survival rate of the culturable, VBNC and resuscitated cells were determined using flow cytometry and SYTO 9/PI as described in Section 2.1. The antibiotic concentration corresponding to obviously sudden reduction of survival rate is the minimum bactericidal concentration (MBC). MBC is identified by determining the lowest concentration of antibiotics that reduce the numbers of initial bacterial inoculum by $\geq$ 99.00 %.

2.4. Gene expression evaluation

Expression of various genes associated with the resuscitation mechanism was quantitatively evaluated by qPCR. The 16S rRNA gene was tested as internal control. Total RNA was collected from bacterial sample using Spin Column Bacteria Total RNA Purification Kit (Sangon Biotech, China). Then, RTIII All-in-One Mix with dsDNase Kit (monad, China) was used to produce cDNA. The 16S rRNA gene was also tested as an amplification internal control. The primers were designed specifically for this study using the primer3plus software. Q-PCR was performed in triplicate using a Bio-Rad CFX96 System (America) in a total volume of 20 μL, which consisted of 10 μL MonAmp™ ChemoHS qPCR Mix (Takara, Japan), 0.4 μL of each primer (10 μmol/L), 2 μL template DNA (cDNA) and 7.2 μL Sterilized ddH_2O. Detailed procedure is provided in SI. Information about these genes and primers is detailed in Table S3.

2.5. Statistical analysis

All experiments were independently performed at least three times and determined statistical significance. Differences are considered significant if $P < 0.05$.

3. Results and discussion

3.1. Bacteria enter VBNC state to escape damage caused by sub-lethal photocatalysis

3.1.1. Formation of VBNC state of bacteria under sub-lethal photocatalysis

To determine whether *E. coli* could be induced into VBNC state under the tested conditions, various strains including ARB (*E. coli* DH5α (MCR) and *E. coli* DH5α (CTX)) and ASB (*E. coli* DH5α) were subjected to sub-lethal photocatalysis for 10 h, and then their culturability and viability were examined. Culturable cell number of all strains gradually decreased with gradually increasing VBNC cell number during sub-lethal photocatalysis (Fig. 1a). After 6 h exposure, culturable cell number of *E. coli* DH5α and *E. coli* DH5α (CTX) decreased from 10^9 to 0 CFU/mL. Longer time (7 h) was required for *E. coli* DH5α (MCR) to completely lose culturability, indicating shallower dormancy degree of *E. coli* DH5α (MCR) than the other two strains under identical condition. However, number of total viable bacteria for all strains did not significantly reduce during sub-lethal photocatalysis (Fig. S3), which can be further confirmed by following results. After 10 h exposure, no bacteria of all strains grew on nutrient agar plates, but most of them retain viability ('green') based on LIVE/DEAD assays (Figs. 1b and S4), indicating that both ARB and ASB could be induced into VBNC state. Specifically, $> 10^8$ CFU/mL of three strains entered VBNC state after 1 h exposure. Finally,

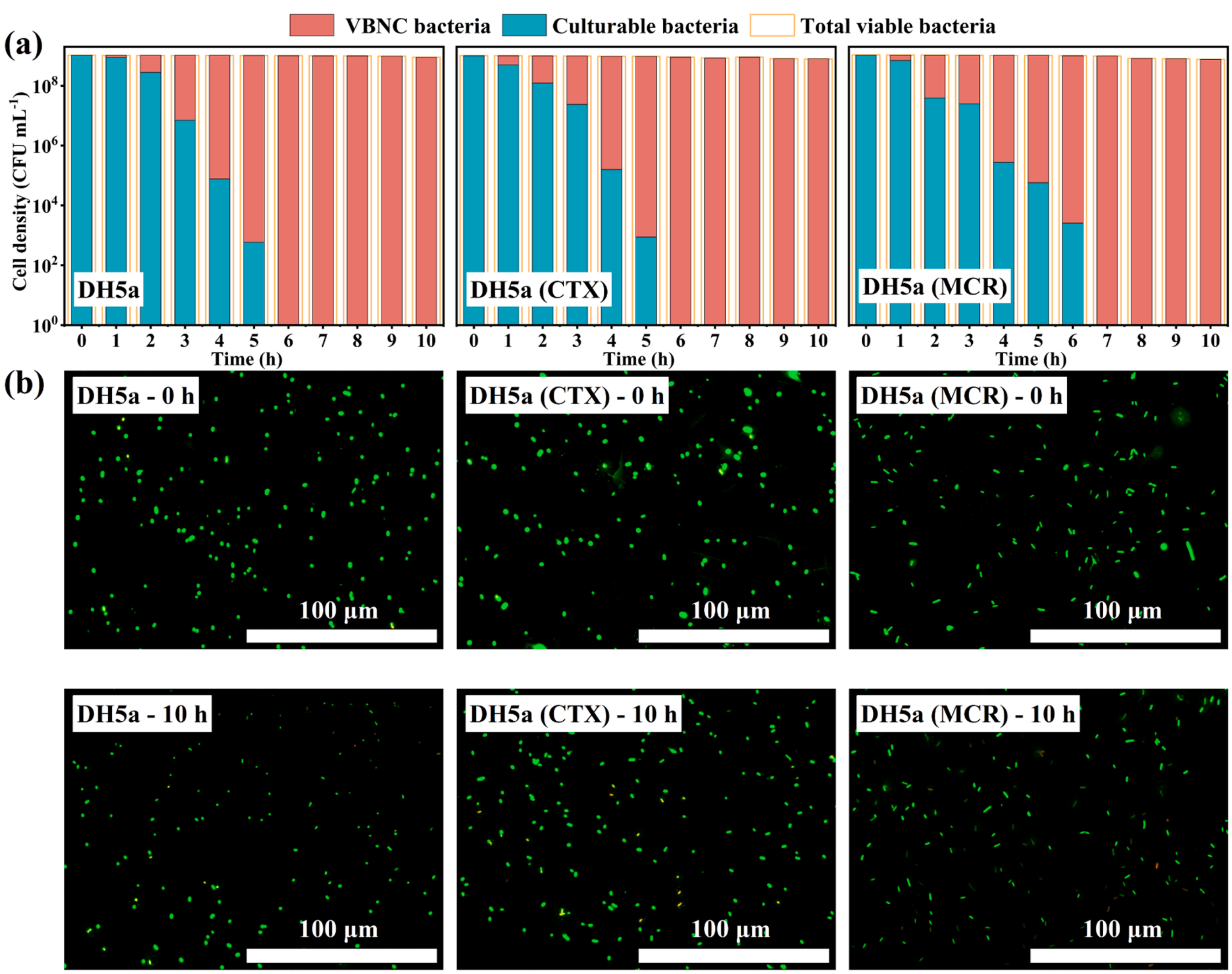

Fig. 1. (a) The number of total viable bacteria (yellow border), culturable bacteria (green bar) and VBNC bacteria (red bar); (b) the live/dead bacteria staining before and after sub-lethal photocatalysis.

all strains entered VBNC state gradually until reaching maximum values. Comparatively, more exposure time required to completely enter VBNC state for *E. coli* DH5α (MCR), mainly due to its higher tolerance to sub-lethal photocatalysis [23]. Further, although exposed for 10 h, VBNC bacteria cannot be completely eliminated, meaning high survival advantage of VBNC bacteria against external stress. This further indicates that threat to human health by the VBNC bacteria might be still preserved, even though some differences exist between WT and VBNC states in physiology and morphology.

3.1.2. Change of physiology and morphology of induced VBNC bacteria

Bacteria develop various strategies to adapt themselves according to the living environment, including change of cell morphology when entering or exiting dormancy [24,25]. Therefore, to further confirm formation of VBNC state, changes of physiological characteristics (including bacterial length, oxidative stress, metabolic activity) for all tested bacterial strains were characterized during sub-lethal photocatalysis.

Based on SEM characterization (Fig. 2), under sub-lethal photocatalysis, some cells lost their membrane integrity (blue arrow, flattened), but VBNC cells still maintained membrane integrity (red arrow). However, shrinkage and size reduction of VBNC cells occurred as compared with their WT. The smaller the specific surface area of a bacterial cell, the lower rate of substance exchange through cell surface, meaning lower metabolic activity [26]. As reported, VBNC *E. coli* maintains a slowed metabolic activity [27,28]. Additionally, ARB, *E. coli* DH5α (MCR) in particular, maintained normal cell morphology better than ASB during sub-lethal photocatalysis, indicating that ARB had higher tolerance. The result about not so significant changes of cell length of *E. coli* DH5α (MCR) accorded to the result that more exposure time needed to completely enter VBNC for *E. coli* DH5α (MCR). Quantificationally, average cell length of *E. coli* DH5α decreased from 2.45 $\pm$ 0.18 to 1.06 $\pm$ 0.12 μm during entering VBNC state (Fig. 3a). Cell length changes of ARB were generally consistent with the trend of ASB, although the decreased extent was less substantially. Further, after entering VBNC state, bacteria had higher cell membrane permeability than WT cells (Fig. 3b), indicating that no visible destruction occurred on VBNC cell bodies, but cell membrane was damaged slightly.

As reported, continuous sub-lethal photocatalysis oxidative stress can damage bacteria [23]. Under different intensity of stress, extent of bacterial damage is diverse, from mild to severe oxidative damage, causing different dormant phenotypes [29]. In turn, these bacteria compelled into a dormant (e.g. VBNC) state can resist oxidative stress [30]. Induction of VBNC bacteria is tightly associated with general stress response system majorly dominated by LysR transcription regulator (OxyR) and RNA polymerase σS (RpoS) [31]. Therefore, whether entering VBNC state being related to damage caused by oxidative stress was further investigated. Higher levels of intracellular ROSs were detected in VBNC than WT cells (Fig. 3c), indicating that redox equilibrium has been destroyed and oxidative stress occurs in VBNC cells. Higher intracellular ROS level was also observed in *E. coli* DH5α than other two tested ARB. This is due to that ARB would induce higher stress responses for enhancing stress resistance, which is a protective response to maintain fitness of bacterial cell membrane [32]. This is also explained why *E. coli* DH5α (MCR) maintained better cell morphology than ASB as abovementioned. Furthermore, to clarify whether VBNC state cell was strong enough to maintain its redox balance, intracellular ROSs of VBNC ARB being induced for 8, 10, and 12 h were determined (Figs. 3d and S5). The sampling points of 8, 10 and 12 h were chosen because Fig. 1a shows that the *E. coli* DH5α (CTX) and *E. coli* DH5α (MCR) strains completely enter the VBNC state after being exposed for 6 and 7 h, respectively. Therefore, the first sampling point was set at 8 h to ensure a stable VBNC state and data collection before any spontaneous recovery occurs. Secondly, the subsequent 10 and 12 h intervals provide a short, evenly spaced gradient to track the dynamics of intracellular ROSs. Intracellular ROSs of VBNC bacteria increased with prolonging time of sub-lethal photocatalysis (Fig. 3d). This means that, with increasing the induced time, the dormancy depth increases with gradually serious damage [33], which will culminate in death even for VBNC cells with higher tolerance due to lethal oxidative damage. This

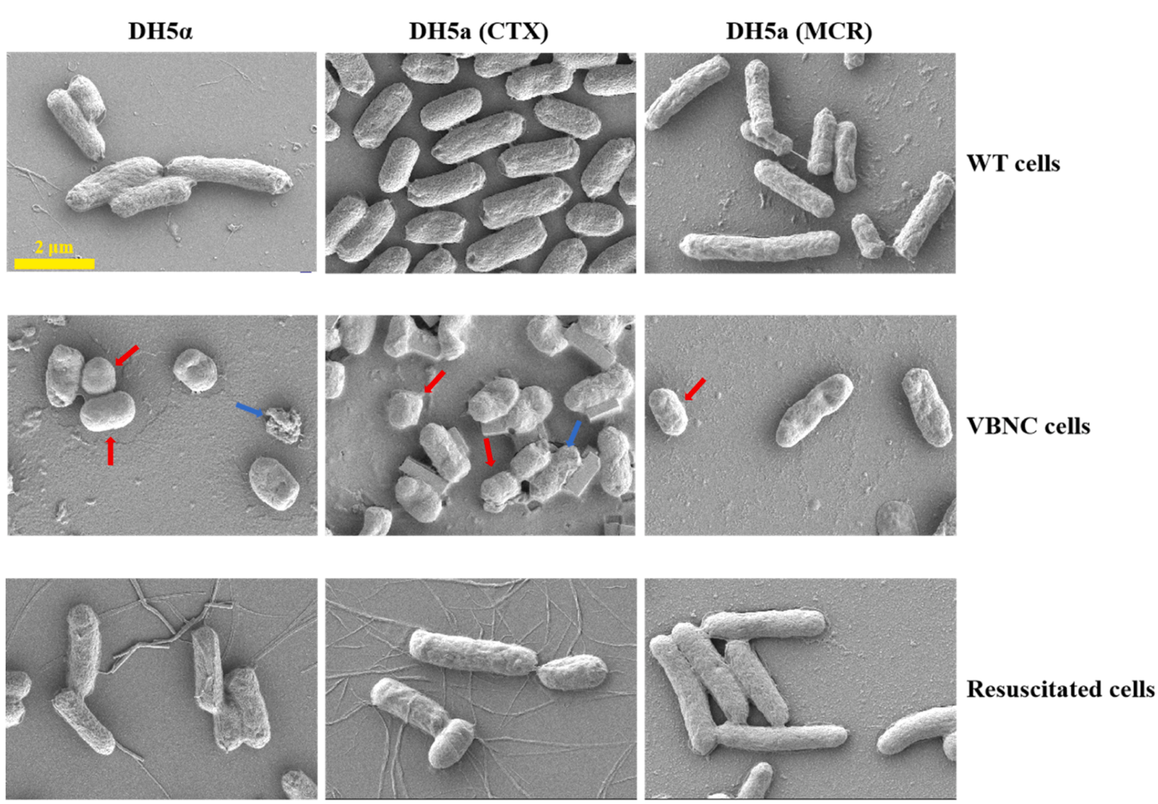

Fig. 2. SEM images of all bacterial strains in different states.

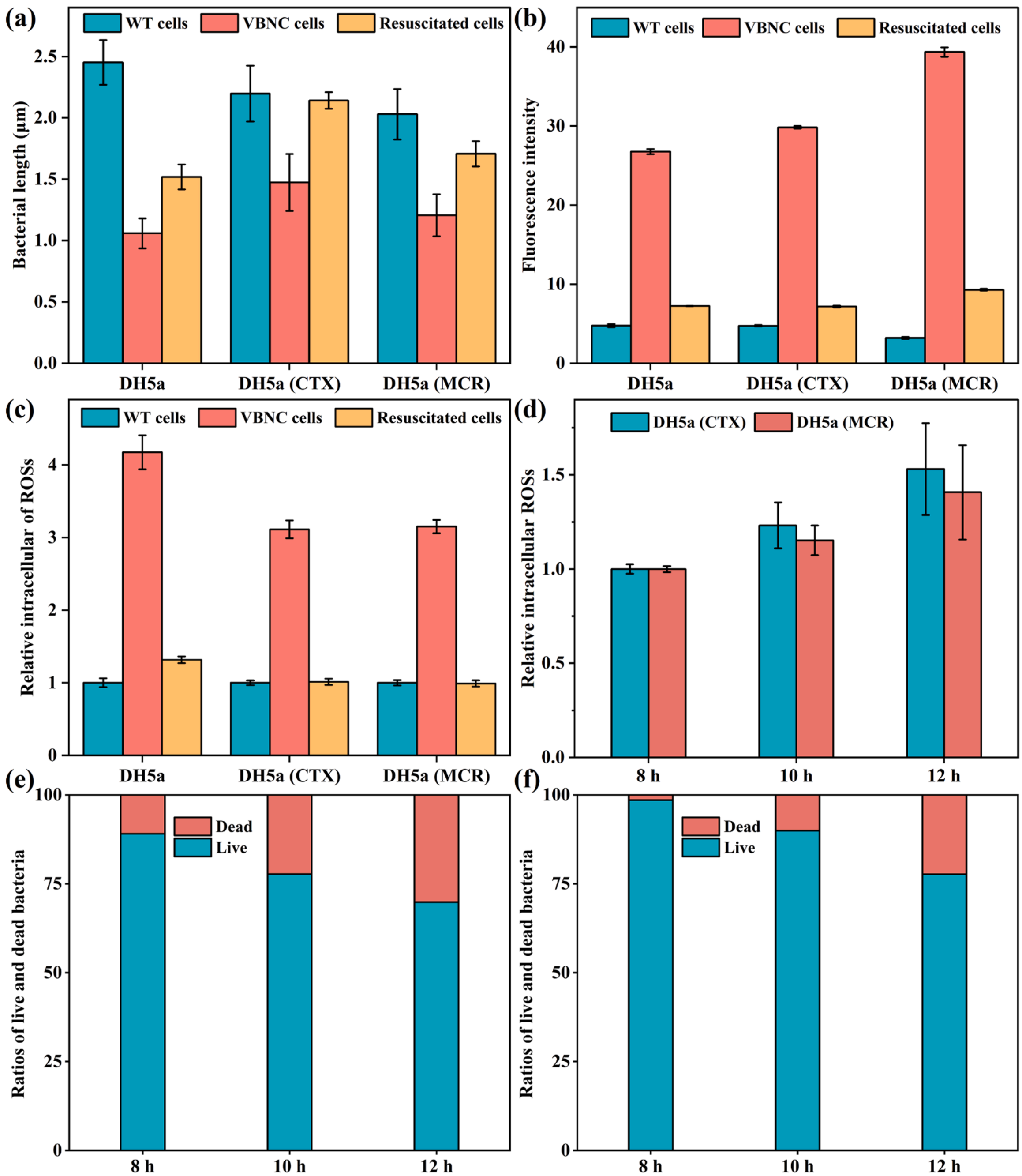

Fig. 3. (a) Bacterial length, (b) membrane permeability, (c) relative intracellular content of ROSs of bacteria in different state; (d) intracellular content of ROSs of VBNC bacteria at different time, and Live/Dead cell ratios of VBNC (e) *E. coli* DH5α (CTX) and (f) *E. coli* DH5α (MCR) induced by sub-lethal photocatalysis at 8, 10, 12 h.

hypothesis was validated by LIVE/DEAD results (Fig. 3e, f), where the dead proportion of VBNC cells increased with the increased exposure time. That is, maintaining redox balance also is key for VBNC bacteria survival.

Oxidative stress also consumes metabolic energy [34]. To clarify VBNC formation mechanism during sub-lethal photocatalysis, the energy metabolism property of them were further examined (Fig. 4a). The relative ATP levels of VBNC cells were significantly lower than those of WT cells. Lower relative ATP levels in VBNC *E. coli* DH5α than the other two strains at same state might be deeper dormancy occurred for *E. coli* DH5α, which well agreed with above-mentioned results. Further, the relative ATP levels of VBNC ARB being induced for 8, 10, and 12 h were studied. With increasing exposure time, ATP depletion became more obvious. Relative ATP level of VBNC CTX (8 h) was approximately 5.3-fold higher than that in VBNC CTX (12 h), while the relative ATP level of VBNC MCR (8 h) was approximately 5.9-fold higher than that in VBNC MCR (12 h) (Fig. 4b). In addition to the changes in energy, the membrane permeability of VBNC bacteria formed at different induction times also varies (Fig. 4c). Compared with the 8 h induction, the membrane permeability of VBNC bacteria induced for 12 h increased by 1.5- to 2.0-fold, and this increase continued to increase over time. The increase in membrane permeability may further affect the subsequent resuscitation of VBNC bacteria. Further, D_2O-labeled Raman Spectroscopy was used to directly detect change of metabolic activity at single-cell level (Fig. 4d). A clear C-D band was observed for WT cell, but not for VBNC cell, suggesting a significantly decrease of metabolic

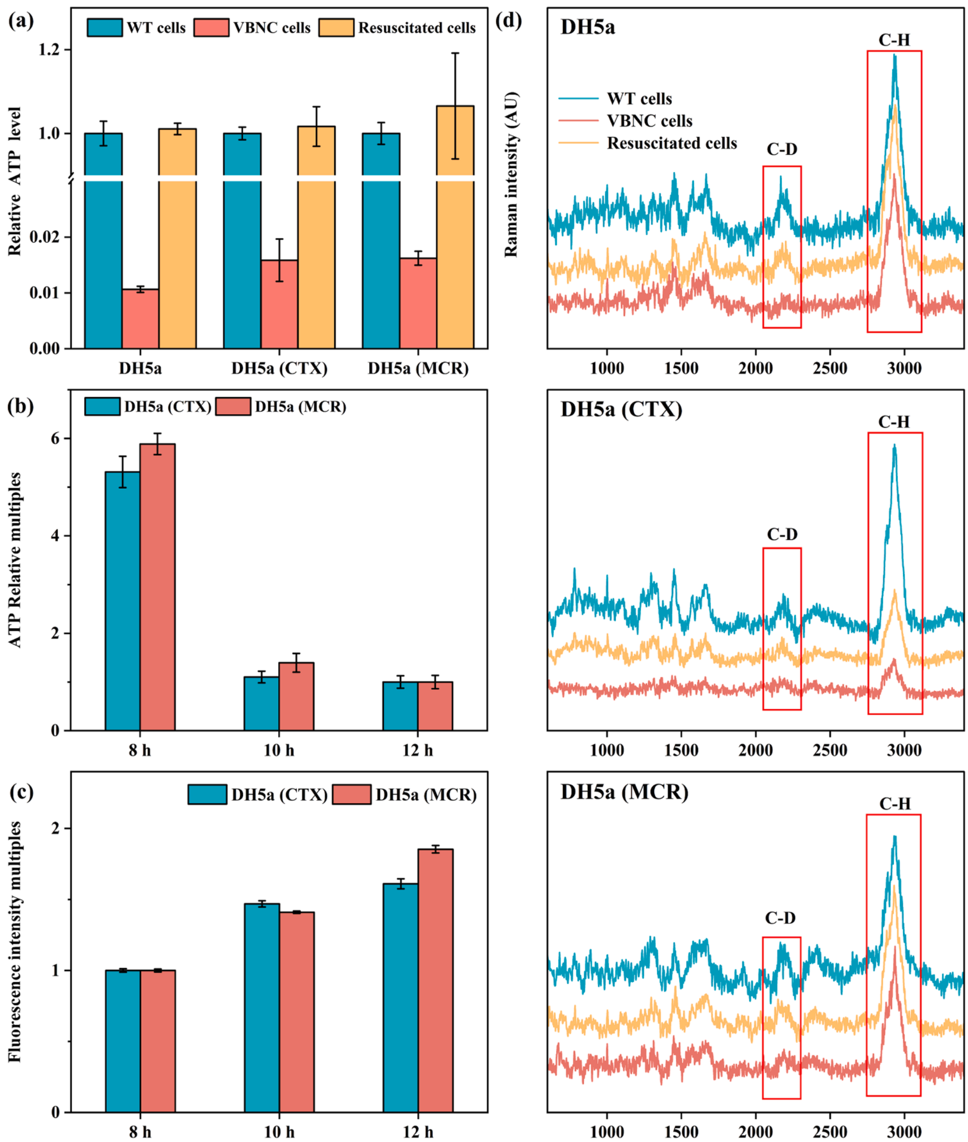

Fig. 4. (a) Relative intracellular ATP level of all bacterial strains in different states; (b) relative intracellular ATP level and (c) membrane permeability of VBNC *E. coli* DH5α (CTX) and *E. coli* DH5α (MCR) induced by sub-lethal photocatalysis at 8, 10, 12 h; (d) the Raman spectra of all bacterial strains in different states.

activity for VBNC cell. Therefore, we concluded that ROS and energy metabolism (ATP and metabolic activity) are essential to enter VBNC state for *E. coli*.

Change of physiology and morphology of the induced VBNC bacteria is only the apparent phenomena. The entering VBNC state may trigger change of other intrinsic properties of bacteria. Among them, bacterial antibiotic resistance is concerned most due to its related to human health [35]. Compared with WT cells, antibiotic resistance of VBNC *E. coli* DH5α, *E. coli* DH5α (CTX) and *E. coli* DH5α (MCR) increased 2-fold for CTX, 14-fold for CTX, 8-fold for PB, respectively (Fig. S6). These indicate that sub-lethal photocatalysis induced VBNC cells could also be a pathway for bacteria to develop antibiotic resistance. As reported, chlorination-induced VBNC bacteria have higher resistance to some antibiotics [11,36]. Therefore, entering VBNC state is a general pathway for some bacteria to achieve higher transient resistance and develop antibiotic resistance.

In all, sub-lethal photocatalysis can induce bacteria entering VBNC state by exerting continuously oxidative stress as demonstrated by losing culturability, reducing cell size, damaging cell membrane, destroying intracellular redox equilibrium, lowering metabolic activity, and increasing antibiotic resistance no matter what kind of ASB or ARB. The longer the exposed time, the deeper the dormancy of VBNC until they were fully inactivated. Noteworthy, antibiotic resistance gene carried by bacteria acts as an ‘enhancement gene’ to improve bacterial survivability [37], and more slowly enter VBNC state during water disinfection. Improved antibiotic resistance of VBNC ASB and ARB demonstrates that dormancy also is a strategy to develop antibiotic resistance for bacteria.

3.2. VBNC cells could spontaneously resuscitate after removing sub-lethal photocatalysis

3.2.1. Spontaneous resuscitation

VBNC bacteria are considered as dormancy, and they can revive to normal state by resuscitation promotion factors (Rpfs) including pyruvate and Rpf protein [38]. Herein, we performed the experiments about Rpfs promoting the resuscitation of VBNC bacteria, including purifying Rpf protein from the culture medium of *Micrococcus luteus*, pyruvate, nutrient broth, L-glucose, L-alanine, heamin to the VBNC bacterial suspension (Fig. S7). However, these additives could not accelerate the resuscitation of VBNC bacteria induced by this study. As reported, VBNC *E. coli* maintains the slowed metabolic activity and also can resuscitate to fully culturable [27,28]. Thus, our induced VBNC bacteria retaining viability has great potential to resuscitate after removal of sub-lethal photocatalysis. To prove this, VBNC cells of all strains were collected after 10 h induction, at which no bacterial colony was observed (Fig. 1a). After removing stress for 10 d, bacterial colony numbers of all strains significantly increased (Figs. 5a and S8), indicating a spontaneous resuscitation of VBNC cells. All VBNC strains showed a reduction in colony size after resuscitation. The colony size of antibiotic-sensitive strain *E. coli* DH5α decreased from 2.0 to 1.3 mm, while ARB *E. coli* DH5α (CTX) and *E. coli* DH5α (MCR) decreased from 2.1 to 1.1 mm and from 1.3 to 0.8 mm, respectively. Furthermore, abundance of resuscitated cells of all strains increased gradually within 20 d and then decreased slightly with time prolongation (Fig. 5b). We inferred that lack of nutrition may contribute to the latter decline of resuscitated cell numbers. Moreover, VBNC *E. coli* DH5α and *E. coli* DH5α (CTX) could resuscitate more hardly than *E. coli* DH5α (MCR) (Figs. S9–S11), probably due to its shallower dormancy as abovementioned. More importantly, some VBNC cells of the same bacterial strains still remain after removing sub-lethal photocatalysis, which agreed with those above-observed results, that dormancy is a dynamic process. Possible reason is that dormancy depth is different for different cells due to their individual difference. That is, whether resuscitation of VBNC cells occurred may depend on their dormancy depth, thus inferring that VBNC bacterial resuscitation may be related to the ability to resist damage and retention of vitality under sub-lethal photocatalysis.

To validate this hypothesis, the resuscitation of VBNC *E. coli* DH5α (MCR) induced by sub-lethal photocatalysis for 8, 10, 12 h was further investigated. VBNC MCR (8 h) and VBNC MCR (10 h) could resuscitate within 5 d, and the former resuscitated faster than the latter, while resuscitation of VBNC MCR (12 h) requires 20 days for resuscitation (Fig. 5c). This is because VBNC MCR (8 h) maintained highest level of vitality, but VBNC MCR (12 h) maintained lowest vitality (Fig. 3f and 4b), which are well agreed with resuscitation of VBNC bacteria inducted with different times (Fig. 5c). Therefore, the proposed hypothesis was confirmed that resuscitation of VBNC cells depends on induction duration (dormancy depth) and ability of different bacteria to resist damage.

Collectively, the induced VBNC bacteria could resuscitate spontaneously without any treatment (e.g. nutritional addition) after removing sub-lethal photocatalysis. Importantly, not all the induced VBNC cells of the same batch can resuscitate simultaneously, demonstrating that occurrence of resuscitation may depend on dormancy depth. This can be further confirmed by the data that, for VBNC cells, the longer the induced time, the harder of bacteria resuscitation, due to increasing damage [33] and more depth of dormancy. That is, the less damaged dormant bacteria could be repaired and resuscitated more easily, whereas seriously damaged ones take longer time to be repaired or even beyond repair [33]. This is also why VBNC MCR (12 h) requires a longer time for resuscitation spontaneously. Additionally, number of resuscitated bacteria increased first and then decreased might be that these resuscitated cells can reenter VBNC state or even die after nutrition lack [12,39].

3.2.2. Resuscitated bacteria showed heterogeneous physiological characteristics

The changes of various physiological characteristics (cell length, colony size, growth rate and antibiotic resistance) for all bacterial strains were further studied during conversion of VBNC to resuscitated state. By comparing with WT cells, the change from resuscitated bacteria return to WT state were also determined.

Based on the SEM characterization (Fig. 2), for all strains, the resuscitated cells had resumed cell membrane integrity and almost recovered to original rod shape from spherical shape of VBNC. These results were further confirmed that cell membrane permeability of the resuscitated cells felled back almost to the same as original WT cells

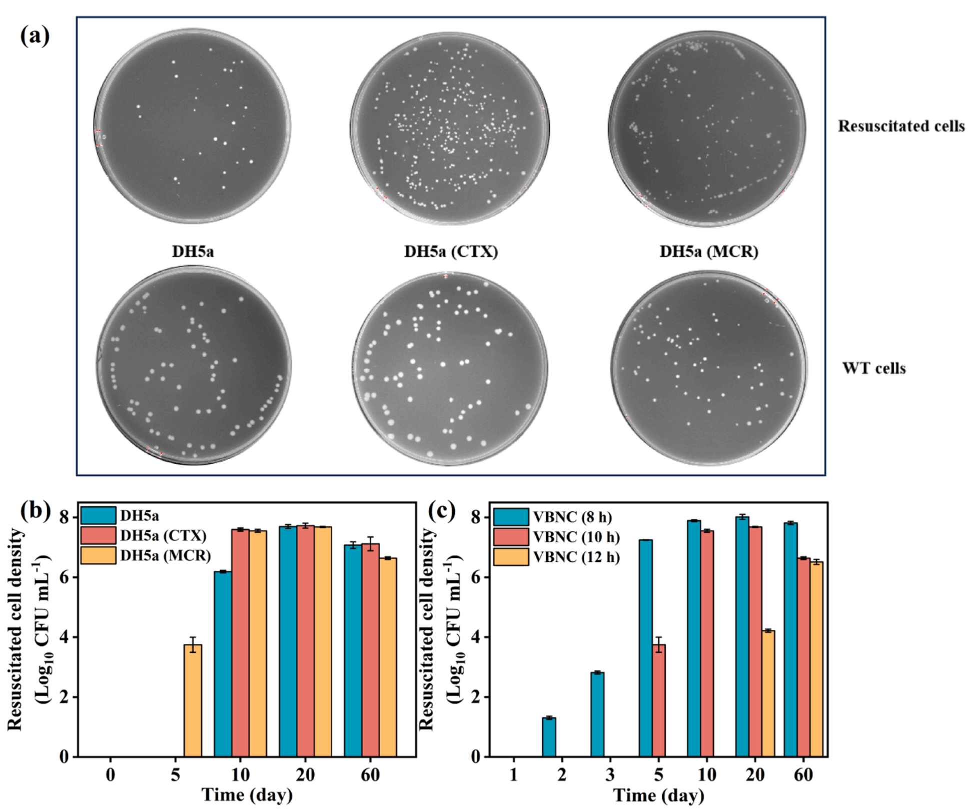

Fig. 5. Monitoring resuscitation of *E. coli* DH5α, *E. coli* DH5α (CTX) and *E. coli* DH5α (MCR); (a) resuscitated cells after sub-lethal photocatalysis removed 10 d and wild type (WT) cells grown on nutrient agar plate for 24 h; (b) the time observed resuscitation of different VBNC bacterial strains; (c) resuscitation of VBNC *E. coli* DH5α (MCR) induced by sub-lethal photocatalysis at 8, 10, 12 h.

(Fig. 3b), suggesting that the damaged membrane was repaired during resuscitation. Quantitatively, average cell length of the resuscitated *E. coli* DH5α was 1.4-time longer than VBNC cells but still shorter than WT cells (0.6-time) (Fig. 3a). Similarly, average cell lengths of resuscitated two ARB were also around 1.4-time longer than their counterpart VBNC cells. Differently, degrees of bacterial length restoration of the tested ARB (>0.8-time) were much higher than that of ASB, further confirming that ARB have higher tolerance than ASB to the stress, resulting in shallower dormancy, and thus subsequently easier resuscitation.

As above-observed, VBNC bacteria could spontaneously restore their culturability. As such, each single bacterial colony size grown on nutrient agar plate for all bacterial strains were investigated. For *E. coli* DH5α, both large (1.52 ± 0.10 mm) and very small colonies (1.06 ± 0.12 mm) appeared after resuscitation, which were distinct from WT cells (2.45 ± 0.18 mm) with homogeneous colony size distribution (Fig. S12a). Similar trend was observed for two ARB strains. For all small colonies, colony size of resuscitated cells of all strains was smaller than WT cells. Differently, for large colonies, colony size of resuscitated ARB was recovered to as large as those of WT cells, whereas resuscitated ASB did not. Herein, we hypothesized that heterogenous colony size may be from different growth rate of resuscitated cells. It is because these VBNC cells were not at same dormancy depth [40] as demonstrated in Fig. 1, where during sub-lethal photocatalysis, number of culturable cells decreased along with increasing VBNC cells over time. Indeed, resuscitation potential of VBNC cells depends on injury degree [14] and dormancy depth [33]. This could explain why heterogeneous physiological characteristics (colony size and growth rate) of the resuscitated bacteria were observed in this study. As such, growth curves of resuscitated cells were recorded using Log Phase 600 Microplate reader, and the growth rates of WT cells were designated as the controls. The resuscitated cells for all strains with large colony grew into exponential phases after 6–8 h, while small colony needed 10–12 h (Fig. S12b–d), indicating that resuscitated cells with small colony size had significantly lower growth rate than large colony.

For antibiotic resistance, during resuscitation of VBNC cell, MBC of *E. coli* DH5α, *E. coli* DH5α (CTX) and *E. coli* DH5α (MCR) decreased 2-fold for CTX, 1.56-fold for CTX, 2-fold for PB, respectively (Fig. S6). The resuscitated cells were picked and grown on an antibiotic-free culture medium to re-proliferate. Then, MBC was measured to verify the heritability of the mutant antibiotic resistance. The results showed that the MBC of the re-proliferated bacterial suspension was not different from that of the resuscitated cells, which at least confirmed the transient heritability of the mutant antibiotic resistance. Besides, MBC of resuscitated ASB cells showed same level as WT cells, while MBCs of resuscitated ARB were still higher than WT cells. These indicate that short adverse environmental exposure may transiently improve antibiotic resistance both for ARB and ASB. Differently, qualitative change in antibiotic resistance did not occur for ASB being exposed to short adverse stress; while ARB could rapidly develop antibiotic resistance [37]. What's more, higher level of transient antibiotic resistance in resuscitated cells may spread further through horizontal gene transfer [36,41]. Therefore, VBNC and resuscitated cells constitute a huge threat to environmental and public health with their higher antibiotic resistance risk.

3.3. Regulating sub-lethal photocatalysis induced VBNC cells and the resuscitation mechanism

3.3.1. Repairing oxidative damage during spontaneous resuscitation

As above-mentioned, during spontaneous resuscitation, the damaged membrane of the bacterial cells could be repaired. Therefore, we further investigated whether bacterial resuscitation is also related to the repairing other oxidative damages. After resuscitation, intracellular ROSs resumed to the same levels as that of WT state for all strains (Fig. 3c), indicating that the destroyed redox equilibrium in VBNC cells has been repaired and recovered. The lower the intracellular ROSs, the easier to resuscitate for VBNC cells due to less damaged. Furthermore, bacteria could recover their cell division ability after repair of damage, like MCR VBNC (10 h). However, bacteria may need more time to repair serious damage, which is why resuscitation of MCR VBNC (12 h) cannot be observed in a short time (10 days). Taken together, repairing oxidative damage caused by sub-lethal photocatalysis could facilitate bacteria to resuscitate from VBNC state upon removing stress. Significantly, after eliminating induction stress, whether or not the dormant cells can resuscitate depending on dormancy depth and damage repairing degree [5,42].

3.3.2. The driving force for the resuscitation: ATP and metabolic activity

To clear the VBNC bacteria resuscitation mechanism, we examined the relative ATP levels of the resuscitated cells. The relative ATP levels of the resuscitated cells completely recovered to the same or even much higher level than those of WT cells, like *E. coli* DH5α (CTX) (Fig. 4a). Based on metabolic activity in the resuscitated cells at single-cell level (Fig. 4d), a clear C-D band suggested that metabolic activity was recovered after stress removal until reaching nearly the same levels as WT cells. Therefore, energy metabolism (ATP and metabolic activity) is also essential to dormant cell to resuscitate, which is further confirmed by resuscitation of *E. coli* DH5α (MCR) VBNC induced for different times (Fig. 5c). That is, time took for resuscitation increased from 2 to 5 day when the ATP level of VBNC MCR (10 h) was approximately one-fourth that of VBNC MCR (8 h). No resuscitation for VBNC MCR (12 h) occurred in a short time (10 days) under ATP depletion condition, compared with VBNC MCR (10 h) with a high ATP level. These data confirmed that ATP and metabolic activity are driving *E. coli* to resuscitate from VBNC state upon stress removal. It is because, in adverse environment, bacteria can adjust their metabolic activity, cell division rate and morphology, which are strategies to improve survivability to response the changing environment [43,44]. Herein, VBNC cells showed lower levels of ATP and metabolic activity than those of corresponding WT cells. This result may be chalked up to the fact that sub-lethal photocatalysis could increase intracellular ROSs and induce *E. coli* to consume significant energy to cope with oxidative stress, resulting in ATP depletion. ATP depletion would induce formation of protein aggregates, further retard bacterial metabolic activity and induce bacteria into VBNC states [45]. Therefore, energy provided for other energy-dependent physiological activities will be lesser. Bacteria might inhibit their division, even lost culturability (subsequently forcing into VBNC state) under limited energy for survival. Translation of proteins related to non-stress is suppressed, and cellular energy store becomes mainly dedicated to stress response, at the cost of other normal cellular processes [34,46]. This is why VBNC bacteria transformed from rod to spherical shape (Fig. 2), which reduces their metabolic activity through smaller cell surface area [10,26]. Once removing sub-lethal photocatalysis, energy to cope with oxidative stress also decreased. The saved energy would drive VBNC cells to restore their culturability [47]. Subsequently, along with repairing oxidative damage during resuscitation, ATP and energy in bacteria can gradually recover to similar level as WT cells. Bacterial growth is recovered with spontaneously restored metabolism [48]. Dormant bacteria could spontaneously switch back into normal state, and reestablish colony after stress removal [49]. These results provide exact support for the importance of intracellular ATP level and metabolic activity to the resuscitation of VBNC cells.

3.3.3. Molecular regulation mechanism of the resuscitation

To confirm the above-obtained conclusion and reveal resuscitation molecular mechanism, the expressions of various genes were further analyzed (Fig. 6). During transition from WT to VBNC state, expressions of genes related to outer membrane proteins (*ompA*, *ompE*, *ompF*), oxidative stress (*oxyR*, *rpoS*, *soxR*, *soxS*, *relA*), DNA damage repair (*envZ*, *mdtA*, *yiaD*, *basS*) and membrane damage repair systems (*mukB*, *radA*, *recF*, *rpoH*, *rcsC*) up-regulated for all strains. All these results further

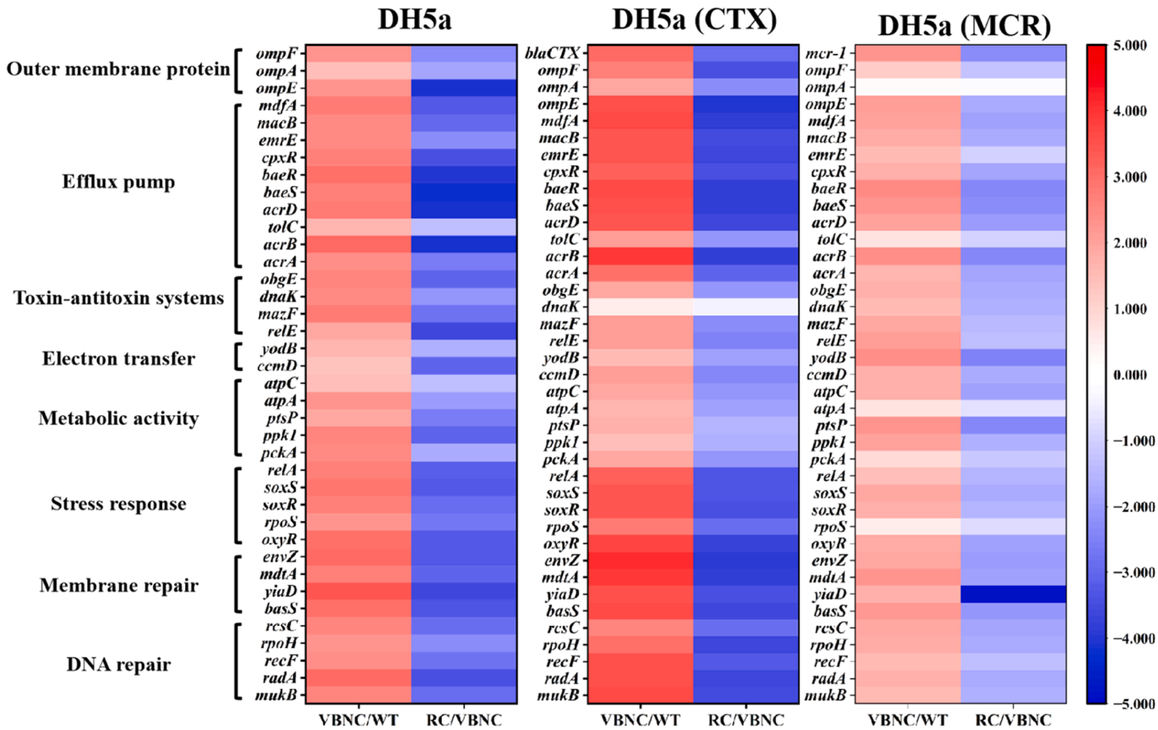

Fig. 6. Gene expression heatmap of target genes involved in resuscitation in *E. coli* DH5α, *E. coli* DH5α (CTX), *E. coli* DH5α (MCR). X-is: Three states; Y-axis left: clusters of target genes and list of genes tested, Y-axis right: the figure legend bar (depicted a blue-red color scale. Red spectrum color indicates up-regulated expression; blue spectrum color indicates down-regulated expression). Results are the mean of independent triplicates.

confirmed that bacterial membrane permeability, ROS stress response, and damage repair of DNA and membrane were all activated during transition from WT to VBNC state. Moreover, expressions of efflux pump related genes (*mdfA*, *macB*, *emrE*, *cpxR*, *baeR*, *baeS*, *acrD*, *tolC*, *acrB*, *acrA*) significantly up-regulated in VBNC cells, indicating antibiotic resistance increased. Besides, *relE*, *mazF*, *dnaK*, *obgE* in toxin-antitoxin system was activated, contributing to forming VBNC cells. Expressions of genes associated with metabolic activity (*ptsP*, *ppk1*, *atpA*, *atpC*, *pckA*), electron transfer (*ccmD*, *yodB*) of VBNC cells all up-regulated. These further confirmed bacterial energy homeostasis is destroyed in VBNC cells due to increased synthesis of ATP and low accumulation of ATP. The energy consumed to resist stress is too much to support other life activities. The protective machinery is activated. That is, to response to sub-lethal photocatalysis, bacteria gradually lost culturability, subsequently entering VBNC state.

As reported, bacteria damaged by sub-lethal photocatalysis can trigger stress-response mechanisms [20]. Here, general stress response (encoded by *oxyR*, *rpoS*, *soxR*, *soxS*), stringent response (encoded by *relA*) and SOS response (encoded by *recA*) in VBNC cells were all triggered. To improve survivability, VBNC cells will cost significant energy to cope with oxidative stress, which disrupts energy allocation balance [50] and sacrifices some unnecessary life activities, including limited growth and suppressed reproduction [51]. That is, VBNC cells reduce ATP consumption by inhibiting DNA replication and cell division as an energy-saving survival mode, which can explain why VBNC bacteria lose culturability. Additionally, increased expressions of efflux pump-related genes were consistent with antibiotic resistance phenotype changes. The oxidative stress-mediated development of bacterial antibiotic resistance has been confirmed by us [37,52]. Lower metabolic activity and up-regulated efflux system may be the key reasons for VBNC bacteria with improved antibiotic resistance [53].

After removing sub-lethal photocatalysis, expressions of these genes almost recovered to their WT's levels for all strains (Fig. 6). It is because bacteria do not need much energy to cope with oxidative stress, subsequently recover the intracellular energy balance. During resuscitation, levels of ATP and metabolic activity increased and bacteria can grow on culture medium again, meaning resuscitation of VBNC cells. We confirmed that repaired oxidative damage and induced changes in energy allocation are driving force for resuscitation of VBNC *E. coli*. Entering VBNC state forms a survival advantage against external stimuli [30]. However, cells can alter their physiology toward resuscitation after removing stress [19]. Moreover, one of the prerequisite conditions for bacteria to resuscitate from VBNC state is restoring energy homeostasis.

4. Conclusions

Effective water disinfection is great important to the quality of water supply and human health. However, microbes develop various strategies to survive including entering dormant states under these unfavorable conditions. Herein, the regulatory formation of VBNC state bacteria induced by sub-lethal photocatalysis and their resuscitation mechanism were explored in-depth from various aspects. Both ARB and ASB *E. coli* can be induced into VBNC state by sub-lethal photocatalysis as demonstrated by losing culturability, reducing cell size, damaging cell membrane, destroying intracellular redox equilibrium, lowering metabolic activity, and increasing antibiotic resistance no matter of ASB or ARB. However, after removing the stress, the induced VBNC bacteria could spontaneously switch back into normal one, growing state and reestablish colony, except for the colony size and antibiotic resistance, by repairing oxidative damage and restoring energy homeostasis in particular, which drive VBNC cells to resuscitate. Concretely, the resuscitated cells showed both large and very small colonies, which may be resulted from different growth rate of resuscitated cells due to various dormancy depth. Notably, antibiotic resistance of VBNC cells of ASB increased, while that of VBNC and resuscitated cel61ls of ARB higher than WT cells. These indicate that short adverse environmental exposure may transiently improve antibiotic resistance both for ARB and ASB. Differently, qualitative change in antibiotic resistance development did not occur for ASB being subjected to short adverse environmental exposure; while ARB could accumulate this property to rapidly develop antibiotic resistance. Therefore, VBNC and resuscitated cells constitute a huge threat to environmental and public health with their higher antibiotic resistance risk. Meanwhile, this study also has some limitations, such as the selection of *E. coli* DH5α, which is an engineered laboratory strain with well-characterized genetic defects, including mutations in the *recA* (DNA repair), *endA*, and *lacZ* (cell cycle regulation) pathways. These defects may affect stress response, DNA repair, membrane dynamics, and overall survivability. Therefore, further research is needed in the future. In addition, the experimental conditions of this study were failed to replicate the complex environment found in actual water treatment systems, so it is recommended that future validation be conducted in water bodies or pilot-scale systems that more closely approximate real-world conditions. In conclusion, this work offers some new perspectives on the formation and resuscitation of VBNC bacteria, which is beneficial for the future environmental control of antibiotic resistance.

Environmental Implication

Bacteria enter VBNC state under stress, which may resuscitate again and pose human health risk. The findings indicate that bacteria can be induced into VBNC state by sub-lethal photocatalysis with increasing antibiotic resistance no matter of antibiotic-resistant or antibiotic-susceptible bacteria. However, VBNC bacteria could spontaneously switch back into normal one, growing state and reestablish colony, except for the colony size and antibiotic resistance, by repairing oxidative damage and restoring energy homeostasis in particular, which drive VBNC cells to resuscitate. As such, VBNC and resuscitated cells constitute a huge threat to environmental and public health with their higher antibiotic resistance risk.

CRediT authorship contribution statement

Guiying Li: Writing – review & editing, Validation, Supervision, Funding acquisition. **Taicheng An:** Writing – review & editing, Validation, Supervision, Conceptualization. **Liu Jiejing:** Writing – original draft, Methodology, Investigation, Formal analysis. **Yiwei Cai:** Writing – review & editing, Validation, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.140041.

Data availability

No data was used for the research described in the article.

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