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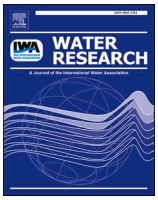

Super-robust synthetic microorganism can get chlorine resistance in advance and transfer their inserted DNA sequence in genome to indigenous bacteria in water

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ABSTRACT

CRISPR-Cas gene editing tools have brought us to an era of synthetic biology that will change the world. Synthetic microorganisms (SMs) have brought enormous economic benefits and will contribute more in the future. Among them, super-robust SMs can overcome the stresses in bioproduction and further increase yield. However, when they are released into the environments, little is known about their fates and risks to human health. In this study, it was found that the gene editing super-robust SM could transfer its inserted DNA sequence in genome to the indigenous bacteria in surface water and showed stronger resistance to chlorine compared with wild-type bacteria. Chlorine disinfection did slight damage on cell membrane of super-robust SM, which decreased ATP leakage and DNA damage, and thereby promoted bacterial survival. Chlorine-injured super-robust SM retained high respiratory activity, and could resuscitate and regenerate. Less damage on super-robust SM cell membrane could prevent chlorine from entering the cells and resulted in lower ROS generation. Its DNA repair system and antioxidant system could still function under high concentrations of chlorine exposure. These findings provided new insights into the fates and environmental risks of SMs as an emerging biological pollutant in water supply system.

1. Introduction

Synthetic biology based on CRISPR-Cas gene editing technologies is changing the world. The development of advanced technologies for reading and writing DNA has resulted in groundbreaking progress in the design, assembly, manipulation of genes, materials, circuits, and metabolic pathways. These progressions have enabled scientists to manipulate biological systems and organisms to a greater extent than ever before (Kiran et al., 2024). The development of synthetic microorganisms (SMs) has promoted the achievement of cost-effective productivity and yield (Adiego-Pérez et al., 2019), which will help biotechnology enterprises develop into a market worth 100 billion dollars by 2025 (Clarke and Kitney, 2016). SMs play a crucial role in the transition to a more sustainable industry to produce food (Korcz and Varga, 2021), feed (Wolf et al., 2021), biomaterials (Hussain et al., 2019), biofuels (Zhang et al., 2021) and pharmaceutical compounds (Daba et al., 2021).

However, like most emerging technologies, opportunities come with potential risks. Industrial fermentation in biotechnology enterprises will generate a large amount of wastewater. For example, producing one ton of citric acid can produce about 50–60 tons of wastewater (Wang et al., 2023). The residual waste liquid from product extraction and equipment cleaning will generate wastewater, which contains a large number fermentation strains, including SMs. The treatment of industrial fermentation wastewater mainly involves suspended particles and nutrient removal and disinfection process (Dutta et al., 2021). However, the disinfection process in wastewater treatment can only inactivate some of the microorganisms, not all of them. In other words, the regulations on the management of synthetic microorganism are missing. Although in order to prevent synthetic microorganisms from entering natural environment, several research strategies in lab have been implemented to establish biological containment, such as inducible systems, auxotrophy and cellular circuits (Torres et al., 2016), containment strategies are not sufficient to prevent synthetic microorganisms escape because of the low evolutionary cost of bypassing or reverting the containment mechanism. Mutation is the main reason for the failure of containment strategies. These biological containment

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strategies have not been applied in the real management to control the synthetic microorganisms. Therefore, synthetic microorganisms can enter surface water with the discharge of industrial wastewater. The leakage of SMs is inevitable. The applications of SMs might go wrong in open environment (Power, 2021). When academic research and commercial applications intentionally or unintentionally released them into the environments, little is known about their fates and the ecological risks.

E. coli has become one of the most widely used chassis cells in the field of biomanufacturing, due to its specific advantages, such as fast growth, easy cultivation, clear metabolic background characteristics and availability of genetic engineering tools (Pontrelli et al., 2018). Nowadays, *E. coli* has been successfully engineered for production of a series of industrially relevant chemicals, including propanediol (Sabra et al., 2016), butandiol (Burgard et al., 2016), isobutanol (Atsumi et al., 2010), L-alanine (Zhang et al., 2007), succinate (Zhu et al., 2014) and so on. However, performances of *E. coli* are always limited by the inhibition of bioproducts (Lian et al., 2016). Due to the role of cell membrane as a protective barrier, membrane damage is considered the fundamental mechanism of inhibitor toxicity (Cao et al., 2017). Numerous studies have attempted to improve the rigidity of cell membrane of *E. coli*, thereby enhancing the tolerance of membrane and the yield of bioproducts (Santoscoy and Jarboe, 2022; Tan et al., 2016). However, the potential ecological risks of these super-robust SMs maybe particularly salient, as they may be more adaptable to some extreme environments. Unfortunately, there is no direct report or research on the ecological risks associated with the release of these super-robust SMs.

Chlorine disinfection has been widely used for drinking water treatment (Lindmark et al., 2022). If genetic modification in super-robust SMs enhancing their tolerance to organic bioproducts increases their tolerance to chlorine disinfectants in advance, it will pose threats to the bio-safety of drinking water. Investigating the chlorine resistance of such super-robust SMs is significant for controlling them in water supply systems. In addition, horizontal gene transfer (HGT) is a major ecological risk of SMs (Kuiken et al., 2014). Several pathogenic bacteria have been detected in source water, such as *E. faecalis, Salmonella* sp. and *Shigella* sp. (Guo et al., 2021). This provides a prerequisite for gene transfer of super-robust synthetic microorganisms. If the inserted gene in super-robust SM, which enhances bacterial robustness, is transferred to these pathogenic bacteria and improves their chlorine resistance, they can pass through the drinking water treatment process and pose a significant risk to human health. Although the probability of HGT is very low, it plays an important role in gene proliferation. For example, HGT is recognized as the primary mechanism for the proliferation of antibiotic resistance in environment (Zarei-Baygi and Smith, 2021). Whether the inserted DNA sequence can spread from SMs to other indigenous bacteria, either through conjugation, transduction or transformation is unknown.

This research focused on a strain of super-robust SM, whose genome for cell membrane composition had been edited to improve its production of bioproducts. Its survival in natural surface water was confirmed. Its resistance to chlorine was characterized, including membrane damage, ATP leakage, culturability, DNA damage, stress response and chlorine inactivation efficiency. Whether gene transfer from super-robust SM to indigenous bacteria occurred in drinking water and whether the tolerance to chlorine increased after receiving the robustness gene were tested. Respiratory activity, as well as resuscitation and regrowth of chlorine-injured super-robust SM were investigated.

2. Materials and methods

2.1. Selected strains in this research

All strains used in this study were listed in Supplementary Table S1. Strain IME was a super-robust SM and derivative of *E. coli* MG1655. Strain IME was obtained by introducing sterols into the cell membrane of *E. coli* MG1655 through inserting an exogenous gene from *S. cerevisiae*. The sterols conferred *E. coli* MG1655 with a robust and stable cell membrane, and improved *E. coli* MG1655 tolerance to a variety of toxic feedstocks and inhibitory products. For example, lignocellulose-derived feedstocks are a class of important renewable resource for biomanufacturing, but they contain several inhibitors, such as hydroxymethylfurfural, levulinic acid and vanillic acid (Ning et al., 2021). Strain IME had a 92 %, 25 % and 50.4 % increase in its specific growth rate relative to the control strain in the presence of 24 mM hydroxymethylfurfural, levulinic acid and vanillic acid, respectively (Sun et al., 2023). Strain IME-GFP was obtained by inserting DNA sequence of green fluorescent protein (GFP) at *maeA* site in strain IME by CRISPR-Cas9 method (Li et al., 2021).

An indigenous bacterial consortium was enriched through using Enterobacteria Enrichment Broth medium and applying surface water as inoculum. The surface water was collected in a tributary of Huangpu River in Shanghai, China.

Bacteria were cultured in Luria-Bertani (LB) medium at 37 °C for 12 h with shaking at 160 r/min, respectively. Bacterial cells were harvested by centrifugation (4000 r/min for 10 min) at 4 °C and washed twice with phosphate buffered saline (PBS, pH=7.4). The cells were then re-suspended into PBS to achieve an initial concentration of approximately 10^6 Colony-Forming Units/mL (CFU/mL) for further chlorine disinfection experiments.

2.2. Survival capability test of super-robust SM in natural surface water

In order to investigate the survival capability of super-robust SM in natural surface water, strain IME and strain MG1655 were inoculated (1 % or 10 %) in surface water, respectively. The natural surface water was collected from Jinze reservoir coming from Lake Taihu, China. The microbial consortia were collected while inoculating for 1, 2, 4, 8 days. The relative abundance of strain IME or strain MG1655 in the collected microbial consortia was detected by qPCR method. The details were shown in Supplementary Text S1.

2.3. Chlorine disinfection experiments

A free chlorine stock solution was prepared by diluting a sodium hypochlorite (NaClO) solution. The stock solution was spiked into the cell suspension to establish target concentration of total chlorine (0, 0.3, 0.6, 0.9, 1.2, 1.5, 2, 4 mg/L). Chlorine disinfection was quenched after 30 min contact time through adding sodium thiosulfate ($Na_2S_2O_3$) solution (0.1 %, 1:1000). The experimental conditions for chlorine disinfection were shown in Table S3.

After chlorine disinfection experiments, samples were collected for further analyzing. Each experiment was performed in triplicate.

2.4. SM characteristics tests after chlorine disinfection

2.4.1. Viable cell counting

Viable cells of super-robust SM and wild-type bacteria after chlorine disinfection were compared by Flow Cytometry (FCM, Cytoflex, Beckman Coulter, USA) with two different fluorescent dyes, including SYBR Green I (SGI) and 7-Aminoactinomycin D (7-AAD).

Samples were diluted with 0.22 μm-filtered PBS to obtain cell concentrations in the range of 10^3–10^5 cells/mL. Then they were stained with SGI+7-AAD and incubated in darkness at 37 °C for 15 min before measurement. The gating strategies for SGI+7-AAD stained cells were shown in Supplementary Fig. S1.

2.4.2. Intracellular ATP leakage assay

The increase of extracellular ATP was regarded as the leakage of intracellular ATP. Extracellular ATP concentrations of super-robust SM and wild-type bacteria after chlorine disinfection experiments were detected by Tecan SPARK microplate reader (Tecan, Groedig, Austria)

with an Enhanced ATP Assay Kit (Beyotime, S0027) (Liu et al., 2021). Samples were centrifuged at 4000 rpm and 4 °C for 10 min. The collected supernatant was filtered (0.22 μm) and then added into a 96-well plate, followed by fast mixing with detecting solution.

2.4.3. Culturable cell counting

The quantities of culturable cells of super-robust SM and wild-type bacteria after chlorine disinfection were determined by heterotrophic plate counts (HPC). Samples were diluted and evenly spread on LB plates. The plates were incubated at 37 °C for 24 h before being counted.

2.4.4. 16S rRNA gene damage assay

The remaining 16S rRNA gene was used to assess DNA damage of super-robust SM and wild-type bacteria after chlorine disinfection. Genomic DNA (gDNA) was extracted using a standard phenol/chloroform extraction protocol as described in our previous study (Miao and Bai, 2021). The copy number of 16S rRNA gene was measured by qPCR (CFX96 Touch System; Bio-Rad, CA, USA) with 515F/909R primers using a HieffTM qPCR SYBR® Green Master Mix kit (YEASEN, China). Full length of 16S rRNA was amplificated by 8F/1541R primers, and quantified for standard curve preparation (Holmes Victor et al., 2006). Details of primers were described in the Supplementary Table S2.

2.4.5. Resuscitation and regeneration assay

After chlorine disinfection, super-robust SM and wild-type bacteria were washed two times by fresh M9 glucose minimal media and 1 % of them were re-suspended in M9 media. The growth curve was monitored in 2 h increments at 600 nm by an automatic Bioscreen C system (Lab systems Helsinki, Finland).

2.4.6. Determination of respiratory activity

5-cyano-2,3-ditoyl tetrazolium chloride flow cytometric measurements (CTC-FCM) was used to detect the respiratory activity of super-robust SM and wild-type bacteria after chlorine disinfection (Créach et al., 2003). The cell suspensions were stained with CTC (Dojindo, Jan), and then incubated in darkness at 37 °C for 30 min. FCM (Cytoflex, Beckman Coulter, USA) was used to calculate the average florescence intensity of each cell to determine the average respiration intensity.

2.4.7. Reactive oxygen species (ROS) measurement

ROS generations in super-robust SM and wild-type bacteria after chlorine disinfection were measured by FCM (Cytoflex, Beckman Coulter, USA) with a DCF-DA/H_2DCFDA-cellular ROS detection assay kit (UE, China) (Xie et al., 2021).

2.4.8. Superoxide dismutase (SOD) activity measurement

Super-robust SM and wild-type bacteria after chlorine disinfection were sonicated at 20 kHz (150 W) for 10 min by an ultrasonic cell cracker (VCX750, Sonics, USA). Then the samples were centrifuged at 12,000 rpm and 4 °C for 10 min, then the supernatant was collected. The SOD activity assay was performed with a WST-1 method assay kit (Nanjing Jiancheng, China), and measured by a microplate reader (Tecan, Groedig, Austria) (Jin et al., 2020). The absorbance value of indicator was 550 nm.

2.4.9. Determination of gene expression level

Expressions of genes related to SOS response (*soxS*), DNA repair (*recA* and *umuD*) and oxidative stress response (*sodA, sodB* and *sodC*) in super-robust SM and wild-type bacteria after chlorine disinfection were assessed by Real-Time qPCR System (RT-qPCR) (CFX96 Touch System; Bio-Rad, CA, USA) with a HieffTM qPCR SYBR® Green Master Mix kit (YEASEN, China). RNA was extracted using RNAiso Plus reagent (TaKaRa, Japan). mRNA was extracted and transcribed to cDNA using Hifair® III 1st Strand cDNA Synthesis Kit (YEASEN, China). The cDNA was used for RT-qPCR analysis. Specific primers used in RT-qPCR were shown in Supplementary Table S2. The housekeeping gene *gapA* was used as a reference gene to normalize mRNA expression (Møller et al., 2016).

2.5. Residual of super-robust SM in tap water after chlorine disinfection

1 % of strain IME and strain MG1655 were mixed with tap water, respectively. The tap water was collected from Shanghai, China. After chlorine disinfection experiments, microbial consortia were collected for further analyzing the absolute and relative quantification of strain IME and strain MG1655 by qPCR. The details were shown in Supplementary Text S1. Residuals of strain IME and strain MG1655 in tap water were also detected by multiple tube fermentation method. The details were shown in Supplementary Text S2.

2.6. Scanning electron microscopy (SEM) observation

After 30 min exposure to 1.5 mg/L initial chlorine, super-robust SM and wild-type bacteria cell were centrifuged at 4000 rpm for 10 min at 4 °C and re-suspended in a 2.5 % paraformaldehyde solution for 6 h. Then the cells were dehydrated in ethanol series (50 %, 70 %, 80 %, 90 % and 100 % ethanol, each for 15 min) and loaded into a critical point dryer (EM CPD 300; Leica, Vienna, Austria) (Fischer et al., 2019). The cells were attached to a large SEM tube and then coated with gold-palladium. Observation and photomicrography methods were conducted by a field emission SEM (S3400II, HITACHI, Japan) at 15 kV voltage.

2.7. Assay of horizontal gene transfer between super-robust SM and indigenous bacteria

Strain IME-GFP and the indigenous bacterial consortium were mixed in 1:1 ratio and then incubated for two weeks at 37 °C and 160 rpm. After incubation, the indigenous bacteria and strain IME-GFP were sorted by BD FACSAria II (BD Biosciences, USA) and analyzed by BD FACSDiva Software. Due to the inserted DNA sequence was linked with kanamycin resistance, a total of 100,000 collected indigenous bacteria cells were loaded into 1 mL sterile PBS solution and spread on LB agar plate containing 50 mg/L Kan. Whether the inserted DNA sequence from strain IME-GFP had transferred to indigenous bacteria was detected by colony PCR and agarose gel electrophoresis. Universal primers 27F and 1492R were used to amplify the 16S rRNA gene to identify the bacterial isolates (Johnson et al., 2019). The amplified nucleotide sequences were used for NCBI BLAST searches (http://www.ncbi.nlm.nih.gov) (Miao et al., 2022).

2.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 20.0, Armonk, NY: IBM Corp). All the results were expressed as means ± standard deviations (SD). For all tests, a p value < 0.05 was considered significant difference.

3. Results and discussion

3.1. Survival of super-robust SM in natural surface water

While *E. coli* represents a model bacterium and one of the most used chassis in biomanufacturing, performance of *E. coli* during biomanufacturing is often limited by stress of bioproducts. To address this deficiency, lots of super-robust *E. coli* chassis that could efficiently tolerate stress of bioproducts has been obtained by synthetic biology modification (Santoscoy and Jarboe, 2022; Sun et al., 2023; Tan et al., 2016). However, the ecological risks of these super-robust synthetic biology chassis leakage may be more severe, as they may be more adaptable to different environments.

To investigate the risks of super-robust SM in drinking water, the first step is to determine whether it can survive in drinking water. An

introduced bacterial invasion experiment was performed to investigate whether genetic modification in super-robust SM would affect their survival in natural surface water. Two invasive bacteria, strain IME and strain MG1655, were added into the natural surface water extracted from the Shanghai Jinzhe reservoir at a rate of 1 % or 10 %, respectively, and incubated for eight days. Both strain IME and strain MG1655 showed similar growth trends in surface water (Fig. 1). Surface water is a mild environment, which does not exert a great pressure or screen for microorganisms. In 1 % invasion treatment, the relative abundance of strain IME and strain MG1655 increased from 1 % to 3.8 % and 4.38 %, respectively. In 10 % invasion treatment, the relative abundance of strain IME and strain MG1655 decreased from 10 % to 1.95 % and 0.78 %, respectively. When growing in surface water, the growth of strain IME showed a slight decline compared with strain MG1655. This slight decline might be due to the higher burden for protein overexpression of inserted gene (Sun et al., 2023). However, this slight growth decline did not affect the survival of strain IME and its potential risk in surface water. High reproductive pressure (10 %) of exogenous bacteria was not conducive to their survival in surface water. This result might be due to the oligotrophic condition of surface water that could not support a large number of exogenous bacteria to grow. However, strain IME could survive stably after invading surface water for at least eight days.

3.2. Super-robust SM gets chlorine resistance in advance

Strain IME was derivative of *E. coli* MG1655 by introducing sterols into the cell membrane of *E. coli* MG1655 through inserting an exogenous gene from *S. cerevisiae*. The sterols conferred *E. coli* MG1655 with a robust and stable cell membrane, and improved *E. coli* MG1655 tolerance to a variety of toxic feedstocks and inhibitory products during bioproduction. When strain IME is released into the natural water environment and extracted into the constructed drinking water supply system, whether it can get the environmental adaptability, such as chlorine resistance in advance?

FCM was used to evaluate the chlorine damage on membrane permeability (Fig. 2A). After 30 min exposure to 0.3, 0.6, 0.9, 1.2, 1.5 mg/L initial chlorine, intact cell concentration (ICC) of strain MG1655 decreased by 19.8 %, 58.5 %, 60.4 %, 92.3 % and 99.2 %, respectively. In contrast, ICC of strain IME decreased by 5 %, 20.5 %, 27.8 %, 35.4 % and 98.9 %, respectively.

Intracellular ATP leakage was detected through measuring the increase of extracellular ATP after chlorine disinfection (Fig. 2B). After 30 min exposure to 0.3, 0.6, 0.9, 1.2, 1.5, 2 and 4 mg/L initial chlorine, the concentrations of intracellular ATP leakage in strain IME were 1.04, 5.84, 9.48, 15.3, 44.4, 49.4 and 202 nmol/L, respectively, while those in strain MG1655 were 2.15, 18.9, 21.6, 39.4, 82.8, 101 and 308 nmol/L, respectively.

The differences of culturability between strain IME and strain MG1655 after chlorine disinfection were analyzed by HPC (Fig. 2C). After 30 min exposure to 0.3, 0.6, 0.9 mg/L initial chlorine, the culturable rate of strain IME decreased to 5.11 %, 1.79 % and 0 %, while that of strain MG1655 decreased to 2.04 %, 0 % and 0 %.

The V4-V5 region of 16S rRNA was selected as the target gene and used to detect DNA damage of strain IME and strain MG1655 after chlorine disinfection by qPCR. Fig. 2D showed the chlorine damage on intracellular DNA of strain IME and strain MG1655. After 30 min exposure to 0.3, 0.6, 0.9, 1.2, 1.5, 2 and 4 mg/L initial chlorine, the damage rates of intracellular DNA in strain IME increased to 3.73 %, 23.8 %, 24 %, 34 %, 36.8 %, 43.2 % and 55.8 %, respectively, while those in strain MG1655 increased to 5.71 %, 25.9 %, 27.7 %, 48.2 %, 54.3 %, 80.8 % and 83.6 %, respectively.

Therefore, the super-robust SM was more resistant to chlorine compared with wild-type bacteria. Improving SM robustness during biomanufacturing would also improve SM robustness to chlorine in water treatment in advance. During chlorine disinfection, super-robust SM suffered lower chlorine damage on cell membrane permeability (Fig. 2A) and less intracellular ATP leakage (Fig. 2B). The differences in intracellular ATP leakage resulted in super-robust SM being in a culturable state, while wild-type bacteria were in a non-culturable state (Fig. 2C). As ATP is a small molecule compound, it is very prone to be leaked when the membrane permeability increases. This is why bacteria quickly lose their culturability and enter viable but non-culturable (VBNC) state when exposed to low concentrations of chlorine. Bacteria living under total chlorine concentration greater than 0.5 mg/L within 30 min are identified as chlorine resistant bacteria (Luo et al., 2021; Miao et al., 2022). After 30 min exposure to 0.6 mg/L initial chlorine, wide-type bacteria entered non-culturable state, while super-robust SM still maintained culturable state (Fig. 2C). Therefore, genetic modification in super-robust SM would transform them from non-chlorine resistant bacteria to chlorine resistance bacteria. Lower permeability damage on cell membrane of super-robust SM reduced the following entry of chlorine into the cells (Ming et al., 2023), which alleviated damage of chlorine to intracellular DNA (Fig. 2D).

The indigenous microorganisms and chemicals in real drinking water may affect the chlorine resistance of bacteria. Le Dantec et al. (2002) found that *Mycobacteria* cultivated in water exhibited significantly higher chlorine resistance than that grown in culture medium, which may be related to the changes of lipids in cells and the secretion of extracellular polymeric substances (EPS). In order to investigate the resistance of super-robust SM to chlorine in actual drinking water, 1 % of strain IME and strain MG1655 were mixed with tap water, and then exposed to chlorine, respectively (Fig. 3). After 30 min exposure to 0.6 and 1.2 mg/L initial chlorine, the inactivation rates on strain IME were 26.2 % and 48.4 %, respectively, while those on strain MG1655 were 56.7 % and 81.5 %, respectively (Fig. 3A). Besides, when exposed to 0.6 and 1.2 mg/L initial chlorine for 30 min, the relative abundance of strain MG1655 in tap water decreased from 1 % to 0.63 % and 0.37 %, respectively, while that of strain IME maintained between 0.92 % and 1

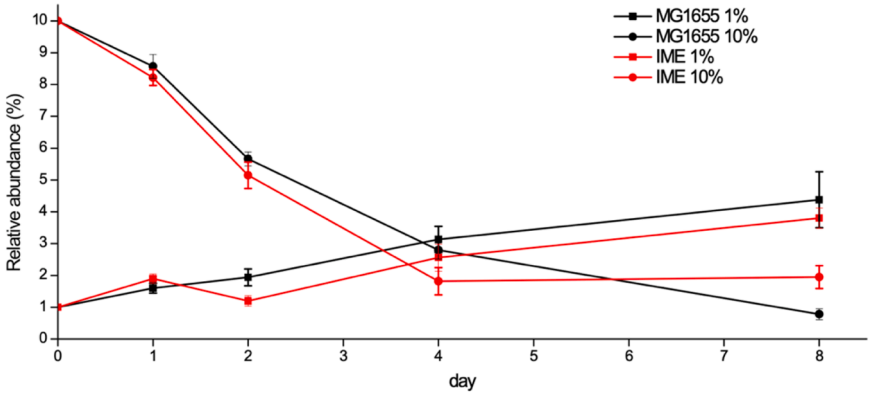

Fig. 1. Survival of strain IME and strain MG1655 in natural surface water.

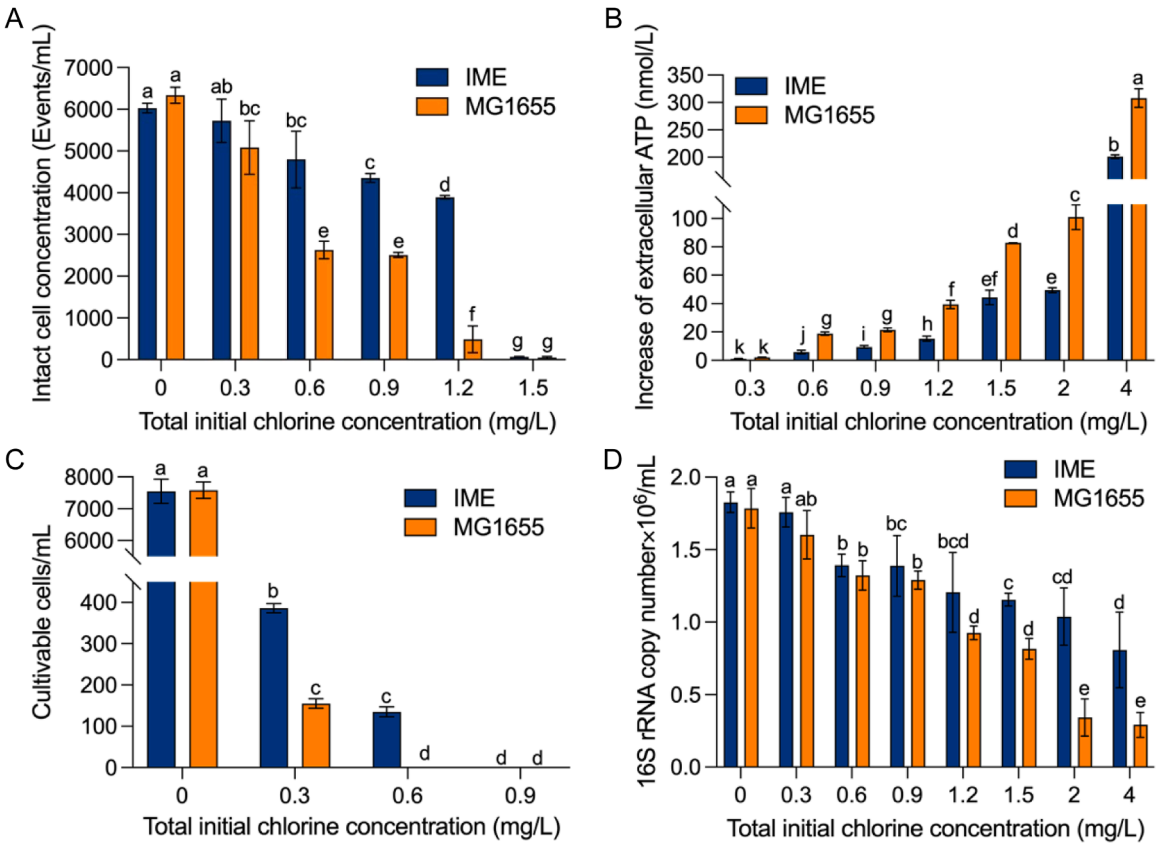

Fig. 2. Viable cell counts (A), increase of ATP leakage (B), culturable cell counts (C) and DNA damage (D) of strain IME and strain MG1655 after 30 min of exposure to various doses of chlorine. Data are means ± SE ($n = 3$). The same lowercase letters represent no significant differences at the 0.05 level. Disinfection experiments were conducted at pH = 7.45 and 28 °C.

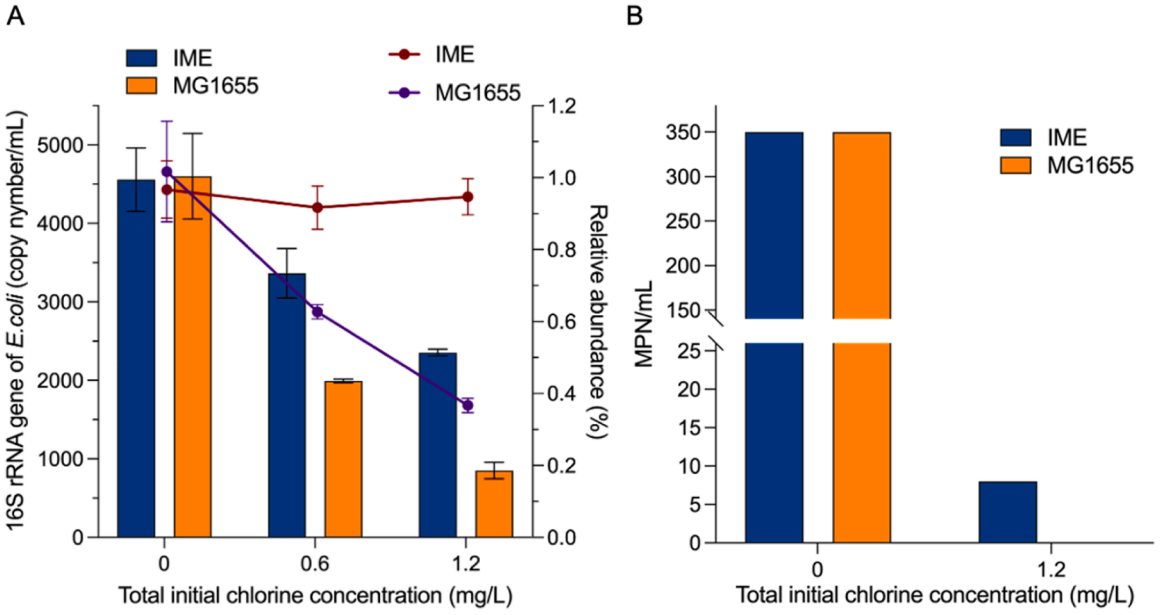

Fig. 3. Relative and absolute abundances of strain IME and strain MG1655 in tap water after 30 min of exposure to 0, 0.6 and 1.2 mg/L initial chlorine detected by qPCR (A). Inactivation of 1.2 mg/l initial chlorine treatment for 30 min on strain IME and strain MG1655 in tap water detected by standard multiple tube fermentation method (B).

% (Fig. 3A). The commonly used concentration for chlorine disinfection in drinking water treatment plants is 1.2 mg/L (Yimer et al., 2022). Therefore, the chlorine disinfection currently used in drinking water treatment plants can not reduce the relative abundance of super-robust SM in drinking water.

What's more, multiple tube fermentation method is the standard

method for detecting *E. coli* in drinking water (Grasso et al., 2000). Standard multiple tube fermentation method was also used to investigate the variation of chlorine inactivation efficiency on strain IME and strain MG1655 in drinking water (Fig. 3B). After 30 min exposure to 1.2 mg/L initial chlorine, strain MG1655 was not detected, indicating complete inactivation. However, 8 MPN/mL of strain IME was still detected. Previous study had isolated an *E. coli* strain from surface water sample taken from Tusciano river, and found that initial chlorine dose as high as 1 mg/L was sufficient to achieve a total inactivation of *E. coli* after 2.5 min contact time (Miranda et al., 2016). Therefore, both of model *E. coli* MG1655 and natural *E. coli* could be eliminated by current chlorine disinfection, and only super-robust SM could escape from current drinking water chlorine disinfection process, which may pose a risk to drinking water biosafety.

3.3. Horizontal gene transfer between super-robust SM and indigenous bacteria in natural surface water

Gene flow is considered as one of the main concerns of SMs when they enter the natural environment. An indigenous bacterial consortium, which was isolated and enriched from one of the Huangpu River tributaries of Shanghai, was mixed with strain IME and incubated to investigate whether the inserted gene in strain IME genome could be transferred to indigenous bacteria. In order to separate strain IME and indigenous bacteria after gene transfer experience, a GFP gene was inserted into strain IME and the strain IME-GFP was obtained (Fig. 4A). After gene transfer experiment, strain IME-GFP and indigenous bacteria were separated by FCM, and the indigenous bacteria were collected (Fig. 4B). An indigenous bacterium colony was found to receive the inserted DNA sequence from strain IME after gene transfer experiment (Fig. 4C). Therefore, the inserted DNA sequence in SM genome which could increase bacterial rubustness to inhibitors in bioproduction could be transferred to indigenous bacterium in surface water. Besides, the indigenous bacterium colony was identified as *Citrobacter freundii* (*C. freundii*). Like *E. coli, C. freundii* also pertains to the family of Enterobacteriaceae. *C. freundii* is a conditionally pathogenic bacterium that primarily affects immunocompromised individuals, leading to various clinical manifestations including pneumonia, meningitis, sepsis, bacteremia and urinary tract infections (Jia et al., 2020). *C. freundii* had been reported that it has a complete type VI secretion system (T6SS) genomic island (Liu et al., 2015). What's more, Borgeaud et al. (2015) have discovered that bacteria can kill other bacteria depending on T6SS, which release their DNA and to be taken up by the killer. The killer can then integrate valuable genes and rapidly evolve (Borgeaud et al., 2015). Therefore, the T6SS in *C. freundii* may endows it with the ability to take up the inserted DNA sequence from strain IME.

In order to investigate whether the chlorine resistance of *C. freundii* would be increased after acquiring inserted DNA sequence from strain IME, three *C. freundii* were isolated from the same bacterial consortium and their chlorine resistance were tested. These three *C. freundii* were named *C. freundii* 1, *C. freundii* 2 and *C. freundii* 3. After 1.2 mg/L initial chlorine treated for 30 min, the intact cells of *C. freundii* with inserted DNA sequence were retained 4 %, while *C. freundii* 1, *C. freundii* 2 and *C. freundii* 3 were completely inactivated (Fig. 4D). The chlorine resistance of *C. freundii* was increased after acquiring inserted DNA sequence from strain IME. The DNA sequences of 16S rRNA gene of isolates were shown in Supplementary Text S3.

3.4. Morphology of the injured super-robust SM after chlorine disinfection

Cell membrane injury is considered as a reliable indicator of bacterial viability loss by most researchers (Van Nevel et al., 2017). In this study, when exposed to 1.5 mg/L initial chlorine for 30 min, both super-robust SM and wild-type bacteria were in a state of complete cell membrane injury (Fig. 2A). Nevertheless, there was a significant difference in the degree of intracellular ATP leakage (Fig. 2B). Hu and Bai (2023) recently discovered that chlorine-injured bacteria could be resuscitated and whether bacteria were completely inactivated

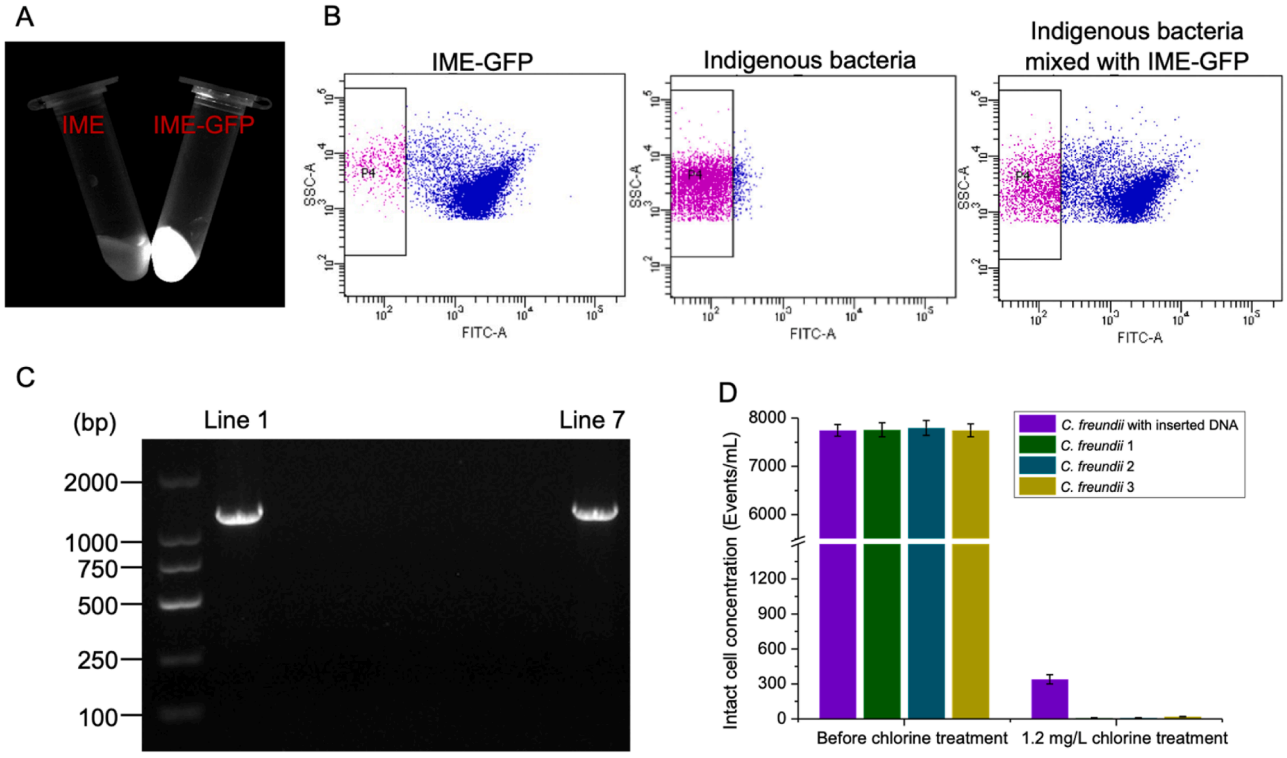

Fig. 4. Images of strain IME and strain IME-GFP under blue light (A). The gating strategy of FCM for sorting indigenous bacteria in mixed culture (B). Electrophoresis gel of engineered DNA sequence measured with PCR extracted from IME (Line 1) and indigenous bacteria after transformation experiment (Line 7) (C). Comparison of viable cell counts before and after 30 min of exposure to 1.2 mg/L initial chlorine (D). Disinfection experiments were conducted at pH = 7.45 and 28 °C.

depended on the degree of injury. In study, SEM was further applied to investigate the degree of injury of super-robust SM after chlorine disinfection.

Morphological characteristics of strain IME and strain MG1655 after chlorine disinfection were determined by SEM examination (Fig. 5A). The untreated strain IME and strain MG1655 exhibited an intact and rod-shaped morphology. After 30 min exposure to 1.5 mg/L initial chlorine, both strain IME and strain MG1655 showed cell wall and membrane damage, indicating that chlorine could react with various cellular components and directly damage their protective layer. The cell morphology of strain MG1655 showed obvious shrinkage, even turning into cell fragments. However, the surface structure of strain IME was only slightly damaged, and the cell morphology remained almost unchanged. Therefore, compared with wild-type bacteria, slight cell membrane injury was observed in super-robust SM when exposed to 1.5 mg/L initial chlorine for 30 min.

3.5. Resuscitation and regeneration of the injured super-robust SM

After chlorine disinfection, injured strain IME and injured strain MG1655 were re-cultured in M9 medium, respectively, and their growth curves were characterized to investigate their resuscitation and regeneration (Fig. 5B). Without chlorine pre-treatment, both strain IME and strain MG1655 reached the exponential phase rapidly after a 4-hour lag phase. After 30 min exposure to 0.3, 0.6, 0.9, 1.2 mg/L initial chlorine, the lag phases of the growth curves of strain IME were extended to 6, 10,

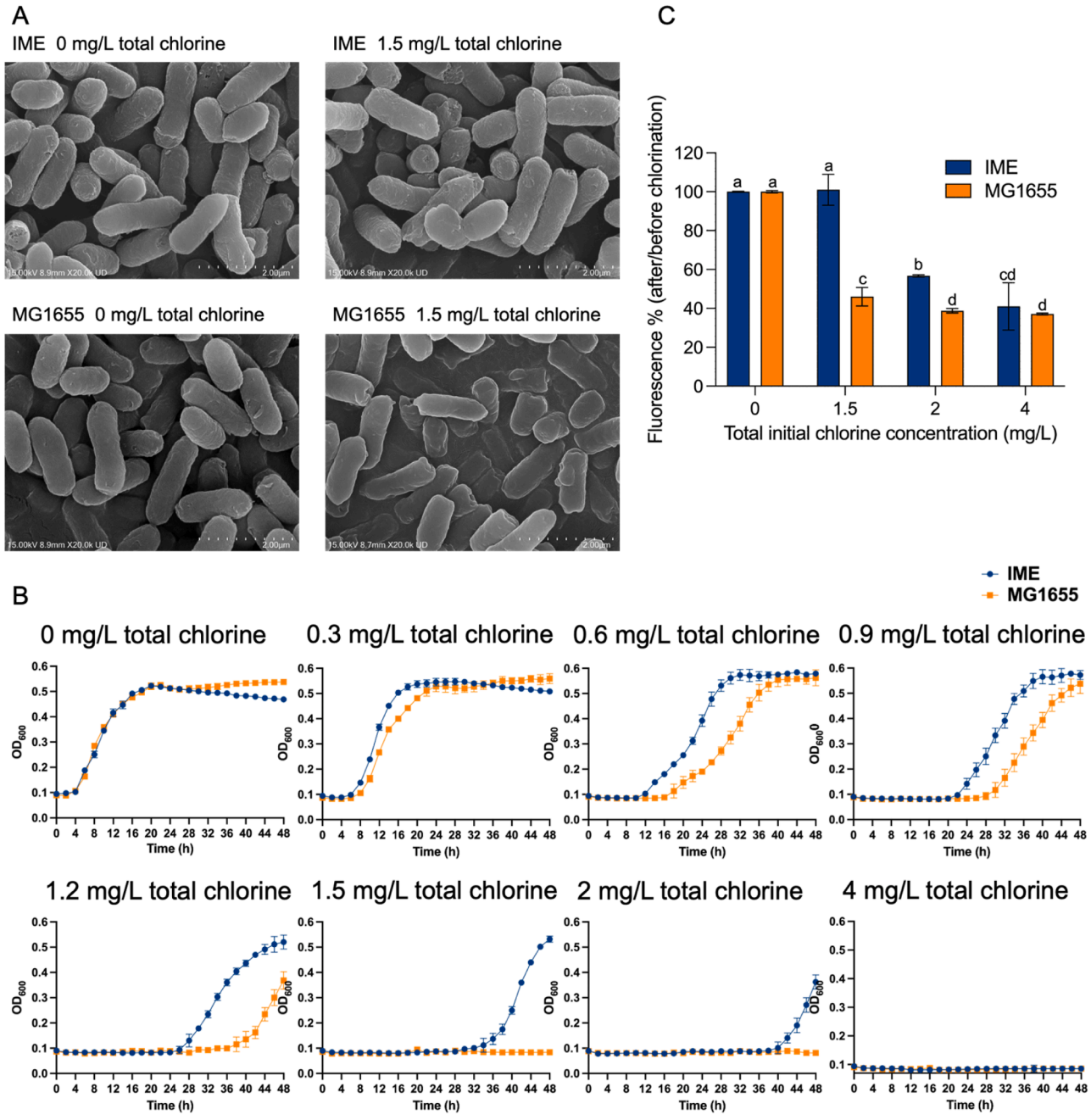

Fig. 5. SEM images of strain IME and strain MG1655 before and after 30 min of exposure to 1.5 mg/L initial chlorine (A). Comparison of the regrowth curves of chlorine-injured strain IME and chlorine-injured strain MG1655 (B). Respiratory activities of chlorine-injured strain IME and chlorine-injured strain MG1655 (C). Data are means ± SE ($n = 3$). The same lowercase letters represent no significant differences at the 0.05 level. Disinfection experiments were conducted at pH = 7.45 and 28 °C.

20 and 26 h, respectively, while those of strain MG1655 were extended to 6, 16, 28 and 36 h, respectively. After 30 min exposure to 1.5 mg/L initial chlorine, both strain IME and the raw strain MG1655 were completely injured (Fig. 2A). However, injured strain IME could be resuscitated and regenerated after 28 h of incubation, while injured strain MG1655 could not be resuscitated within 48 h (Fig. 5B). This result further indicated that after 30 min exposure to 1.5 mg/L initial chlorine, although both strain IME and strain MG1655 were injured, strain IME might only be in a damaged state and could be resuscitated, while strain MG1655 had been completely inactivated and could not be resuscitated. When the initial chlorine concentration increased to 2 mg/L, strain IME still could be resuscitated after 36 h of incubation. The resuscitation potential of chlorine-injured super-robust SM was higher than that in chlorine-injured wild-type bacteria. Bacterial regrowth after water disinfection, which will lead to the deterioration of treated water, poses sever risks to public health. Drinking water residence time in distribution networks is sufficient for chlorine-damaged super-robust SM to resuscitate and regenerate.

3.6. Respiratory activity of the injured super-robust SM

After 30 min exposure to 0, 1.5, 2 and 4 mg/L initial chlorine, the respiratory activities of untreated and injured cells were measured by CTC-FCM (Fig. 5C). Although both strain IME and strain MG1655 had been completely injured after 30 min exposure to 1.5 mg/L initial chlorine (Fig. 2A), strain IME still maintained a high level of respiratory activity (approximately 100 % of untreated cell), while the respiratory activity in strain MG1655 reduced to less than 50 % of untreated cell (Fig. 5C). As the initial chlorine concentration increased to 2 mg/L, the respiratory activity in strain IME still maintained more than 50 % of untreated cell. 1.5 mg/L initial chlorine treatment was the threshold concentration to strain MG1655, at which point, its respiratory activity stabilized at the lowest level (lower than 50 % of untreated cells). The respiratory activity reflects the redox ability of the electron transport chain, and electron transfer is an essential physiological process in viable cells (Guo et al., 2019). Chlorine disinfection damages the cell membrane, where the respiratory electron transport chain is located. Less damage on cell membrane of super-robust SM protected the integrity of the electron transport chain and the normal delivery of electrons. As a result, energy in chlorine-injured super-robust SM was sufficient for basic metabolic activity and assimilating various organic compounds from environment, which supported its resuscitation and regrowth.

3.7. Stress response of super-robust SM during chlorine disinfection

When bacteria are exposed to external environmental stimuli or adverse factors, their internal oxidative stress response mechanism will be triggered, resulting in ROS production for self-protection (Guo et al., 2023). However, excessive ROS will damage bacteria themselves, including their DNA, RNA and proteins, leading to bacterial inactivation (Du et al., 2020). In this study, both strain IME and strain MG1655 showed a significant increase in intracellular ROS generation when exposed to 0.3–4 mg/L initial chlorine (Fig. 6A). Maximum ROS generation was detected under 4 mg/L initial chlorine treatment, where the ROS levels of strain IME and strain MG1655 increased by 15 and 20 times, respectively. Compared to strain MG1655, strain IME produced lower ROS during chlorine disinfection. This might be due to the cell membrane of super-robust SM could prevent part of chlorine from entering the cells.

In order to avoid being damaged by endogenous or exogenous chemical substances, bacteria form an antioxidant system to maintain a

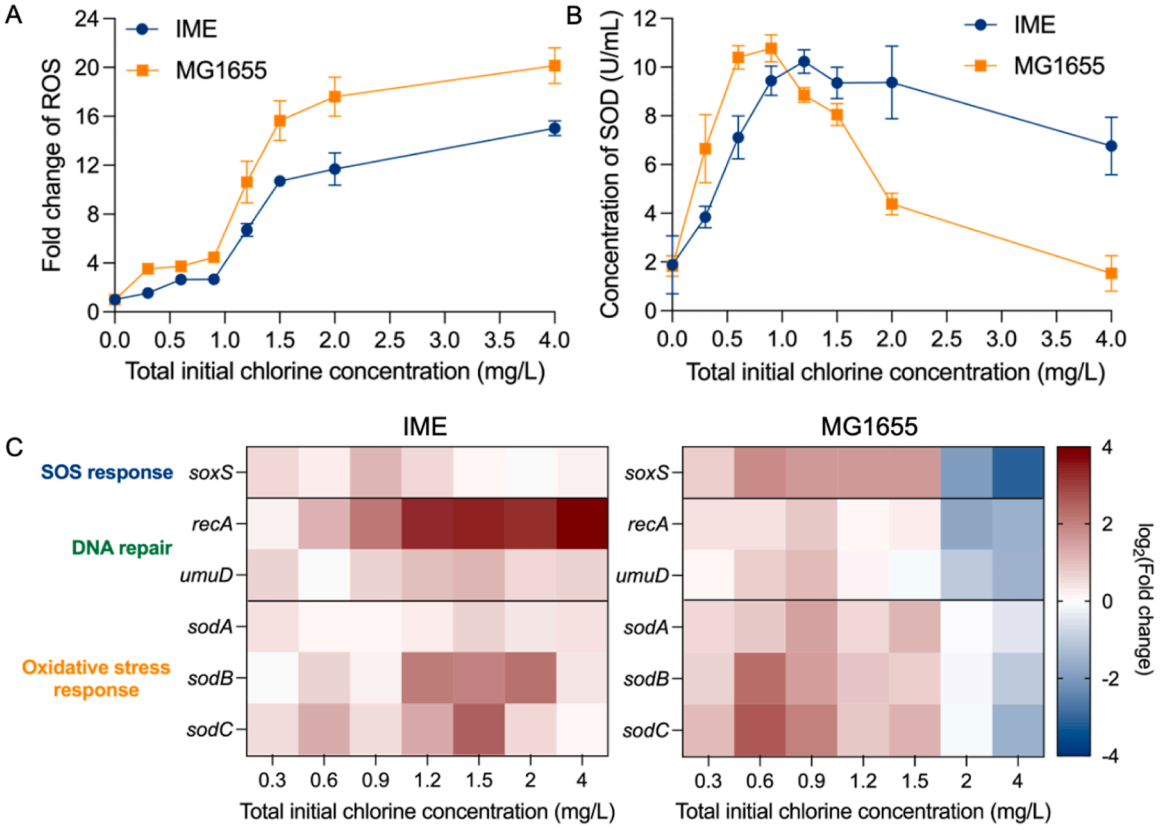

Fig. 6. Fold changes of ROS generation (A), SOD production (B) and the expression level of genes related to SOS response, DNA repair, as well as oxidative stress response (C) between strain IME and strain MG1655 after 30 min exposure to different concentrations of chlorine. Disinfection experiments were conducted at pH = 7.45 and 28 °C.

dynamic balance in generation and clearance of ROS (Halliwell et al., 1995). SOD is an important antioxidant enzyme in nearly all living cells (Yang et al., 2023). Fig. 6B showed the changes of SOD activity in strain IME and strain MG1655 during chlorine disinfection. Both strain IME and strain MG1655 showed an increase in SOD activity when exposed to 0.3 to 4 mg/L initial chlorine for 30 min, except that SOD activity in strain MG1655 when exposed to 4 mg/L of initial chlorine for 30 min. At lower chlorine exposure concentrations, the activity of SOD increased with increasing chlorine exposure concentrations. This indicated that the bacterial antioxidant system exhibited defensive behavior to combat the oxidative damage of ROS. However, as the chlorine concentration continued to increase, excessive ROS attacked the intracellular structure of bacteria, gradually disrupting the bacterial defense system and leading to a decrease in SOD activity. Interestingly, when the exposure concentration of chlorine was below 0.9 mg/L, the SOD activity in strain MG1655 was higher than that in strain IME. However, when exposed to high concentration of chlorine (1.2 to 4 mg/L), the SOD activity in strain IME was higher than that in strain MG1655. The maximum SOD activity in strain MG1655 was detected when exposed to 0.9 mg/L of initial chlorine, while that in strain IME was detected when exposed to 1.2 mg/L of initial chlorine. This was because that under low concentration of chlorine treatments (< 0.9 mg/L), super-robust SM generated lower ROS (Fig. 6A), leading to lower activity of SOD activation (Fig. 6B). When exposed to high concentrations of chlorine (> 1.2 mg/L), less damage on DNA allowed the antioxidant system of super-robust SM still function to clear excess ROS and promote cells survival, while the antioxidant system of wild-type bacteria was destruction. Although genetic modification in super-robust SM did not change their antioxidant system, the strong rigidity of the cell structure protected it to function even in high concentration of chlorine treatment, which would bring great challenges to water disinfection.

Differences in antioxidant system genes expression between strain IME and strain MG1655 during chlorine disinfection were compared based on RT-qPCR (Fig. 6C). Under low concentrations of chlorine exposure (< 0.9 mg/L) for 30 min, up-regulation levels of SOD gene (*sodA, sodB* and *sodC*) transcription in strain MG1655 were higher than those in strain IME. However, when exposed to high concentrations of chlorine (1.2 to 4 mg/L) for 30 min, the expression levels of genes of SOD in strain IME were higher than those in strain MG1655. The transcriptional levels of SOD production genes were consistent with the result of SOD activity assays.

The production of ROS also induces SOS response and DNA repair (Zhang et al., 2024; Zhao et al., 2025). The expression of genes related to SOS response and DNA repair were measured by RT-qPCR (Fig. 6C). Obviously, the repression level of *soxS* was higher in strain MG1655 compared to strain IME when treated by different concentration of chlorine (0.3–1.5 mg/L). The upregulation of *soxS* further indicated that higher ROS levels exited in strain MG1655, which offered enhanced protection against ROS attacks. However, when exposed to high concentration of chlorine (more than 2 mg/L), DNA in strain MG1655 was severely damaged, and its defense systems were disrupted. On the contrary, *recA* and *umuD*, which are genes relevant to DNA repair, showed increased expression levels in strain IME during chlorine disinfection, even exposed to higher concentration of chlorine (more than 2 mg/L). The more rigid cell membrane prevented chlorine from entering the cell, allowing strain IME to initiate DNA repair to reduce the damage of chlorine to DNA quickly during chlorine disinfection.

4. Conclusion

This study demonstrated that gene modification in super-robust SM gave bacteria chlorine resistance in advance and the inserted DNA sequence from super-robust SM could be transferred to indigenous bacterial in natural surface water. During chlorine disinfection, less damage was posed on the cell membrane and DNA of super-robust SM, which decreased ATP leakage and promoted the survival of super-robust SM. After chlorine disinfection, injured super-robust SM still maintained high respiratory activity, enabling it to resuscitate and regrow. What's more, super-robust SM generated lower ROS and its antioxidant system could still function even under high concentration of chlorine. These enabled the super-robust SM to escape from chlorine disinfection in drinking water treatment. This report provides new insights into the potential ecological risks of super-robust SM, which are being applied more and more widely in the biomanufacturing economy. It may become an emerging pollutant in the future and requires more attention from the world.

CRediT authorship contribution statement

Xuejing Huang: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zaigao Tan:** Resources, Methodology. **Jiayu Wei:** Resources, Methodology. **Xiaohui Bai:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123594.

Data availability

Data will be made available on request.

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