Identification and inactivation of *Gordonia*, a new chlorine-resistant bacterium isolated from a drinking water distribution system

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ABSTRACT

Chlorine-resistant bacteria threaten drinking water safety in water distribution systems. In this study, a novel chlorine-resistant bacterium identified as *Gordonia* was isolated from the drinking water supply system of Jinan City for the first time. We examined the resistance and inactivation of the isolate by investigating cell survival, changes in cell morphology, and the permeability of cell membranes exposed to chlorine. After 240 min chlorine exposure, the chlorine residual was greater than 0.5 mg L^{-1} and the final inactivation was about 3 log reduction, which showed that the *Gordonia* strain had high chlorine tolerance. Flow-cytometric analysis indicated that, following sodium hypochlorite treatments with increasing membrane permeability, culturable cells enter a viable but nonculturable state and then die. We also investigated the inactivation kinetics of *Gordonia* following chlorine dioxide and ultraviolet radiation treatment. We found that these treatments can effectively inactivate *Gordonia*, which suggests that they may be used for the regulation of chlorine-resistant microorganisms.

Key words | chlorine-resistant bacteria, disinfection, drinking water distribution system, *Gordonia*

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HIGHLIGHTS

- A new chlorine-resistant bacterium was isolated from the drinking water distribution system.
- The isolate was identiïed as Gordonia JN724 and it was sensitive to chlorine dioxide and UV radiation.
- Flow cytometry provides key data to evaluate the membrane integrity and detect the viable but non-culturable cells in *E. coli* inactivation by chlorine.

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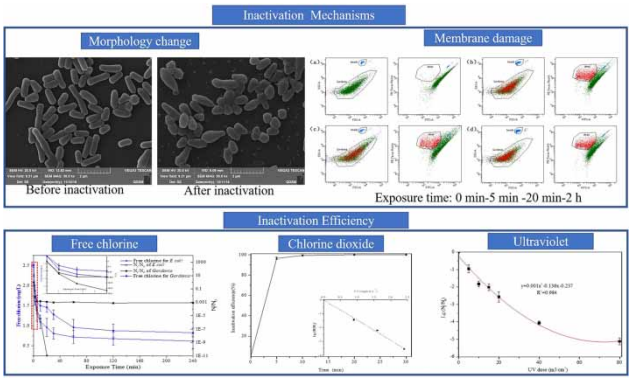
GRAPHICAL ABSTRACT



INTRODUCTION

With the emergence of disinfectant-resistant microorganisms, concerns over the safety of water supplies have increased. At present, chlorine treatment is a universally used technology owing to its reliability and relatively low cost. Chlorine-based disinfectant has been used in the forms of free chlorine and chlorine compounds – e.g., liquid chlorine, hypochlorite, and bleaching powder – in disinfection processes for water plants. Yet excessive chlorine use also promotes selection for chlorine tolerance. To ensure that disinfection is sustained, high chlorine concentrations must be maintained in finished water. An environment with surplus chlorine in water distribution systems provides survival selective pressure and selects for disinfectant-resistant cells (Falkinham 2015).

An increasing number of studies have shown the inefficacy of chlorine in pathogenic microorganism control in aquatic environments, especially in wastewater systems. Not only have *Citrobacter* and many *Bacillus cereus* species been isolated from wastewater treatment plants (Mir *et al.* 1997; Paes *et al.* 2012; Mojisola & Anthony 2017), but various reports have confirmed that microbial biomass decreases with increases in disinfectant-resistant bacteria after wastewater disinfection treatment (Diehl & Lapara 2010; Coronel-Olivares *et al.* 2011; Burch *et al.* 2013; Mojisola & Anthony 2017).

Chlorine-resistant bacteria have also been discovered in drinking water, which threatens public health. Some chlorine-resistant bacteria are pathogenic or conditionally pathogenic, such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Falkinham *et al.* 2001; Chen *et al.* 2012; Gomes *et al.* 2016). Additionally, chlorine-resistant bacteria have led to the regeneration of microbes in the urban pipe network and reduced the biological stability of drinking water. Yet studies on chlorine-resistant bacteria in the water supply system remain scarce compared with those focusing on sewage treatment systems. In particular, few studies have focused on the isolation of chlorine-resistant planktonic bacteria in urban water supply systems (Goncharuk *et al.* 2014).

Moreover, the quantification of microbes has traditionally been performed using plate count methods, but these have great limitations in the detection of chlorine-resistant bacteria. In fact, bacteria may enter a viable but non-culturable (VBNC) state and not be detected by traditional plate count methods when exposed to chlorine-based disinfectant. Consequently, the health risk of chlorine-resistant bacteria may be underestimated. The presence of VBNC bacteria following the disinfection process has been confirmed in previous studies (Li *et al.* 2014; Zhang *et al.* 2018). VBNC cells were found to remain animate as a result of cellular metabolic activity but were unable reproduce and form colonies in conventional culture (Oliver 2000). Hence, they may go undetected during routine colony counting. These bacteria can restore their reproductive capacity under appropriate conditions, and a considerable proportion of chlorine-resistant

bacteria can enter the VBNC state under chlorine stress (Zhang *et al.* 2018). Therefore, detection methods for microorganisms in different states must be employed during the disinfection process. Recent flow-cytometric technological advancements provide rapid and accurate data for bacteriological determination. A cultivation-independent approach using flow cytometry (FCM) in combination with fluorescent probe technology allows for the enumeration and functional classification of the physiological state of bacteria at the single-cell level (Hammes *et al.* 2011). All of the bacteria, including VBNC bacteria during the disinfection process, can be collected and detected by FCM.

In China, the Yellow River flows through nine provinces, and it is one of the most important water sources in the basin. To our knowledge, there have been no reports on chlorine-tolerant bacteria in the water supply system of the Yellow River Diversion Project. In this study, we examined a method for screening and separating chlorine-resistant bacteria in the drinking water distribution system taking Yellow River as water source, and we obtained a chlorine-resistant bacterium (*Gordonia* JN724) identified as belonging to the genus *Gordonia*. Some members of the genus *Gordonia* are opportunistic pathogens that can cause respiratory tract diseases and other related illnesses. The abundant reproduction of *Gordonia* with pigment may lead to high colony counts, color, and turbidity in tap water if environmental conditions are suitable. Moreover, as there have been no reports on the chlorine resistance of *Gordonia* in aquatic environments, we thoroughly investigated it here. We used *Escherichia coli,* which is relatively sensitive to chlorine, as a comparison strain for chlorine-resistance analysis. Analytical methods used in this study included flow cytometry, scanning electron microscopy, and heterotrophic plate counts (HPC). To explore the effective countermeasures, we examined the inactivation of *Gordonia* JN724 via aqueous chlorine dioxide (ClO_2) and ultraviolet (UV) radiation.

METHODS

Isolation of the bacterial strain

Gordonia JN724 was isolated from tap water samples of water distribution system taking Yellow River as water source. Tap water was collected in triplicate along the distribution pipeline of the Quehua water treatment plant in Jinan City, China. The sampling site was 10 km from the water treatment plant. The sampling procedures were in accordance with standard examination methods for water collection and preservation (GB/T 5750.2-2006). Two liters of water were collected in pre-cleaned and sterilized glass bottles, and the samples were refrigerated during transportation and storage. The physical properties of the collected water samples are summarized in Table 1. Membrane filtration was used to isolate the bacterial cells within 4 h. The target water sample of 1 L was filtered with a polycarbonate membrane filter (0.22 μm, Millipore, UK) for cell harvesting. After filtration, the membrane was placed into a tube with 5 mL of phosphate buffered saline (PBS; 0.1 M, pH = 7.2). The bacterial cells attached on the membrane were separated out by shaking on a NP-30S vortex shaker (Suzhou Jiulian Technology Co. Ltd, Suzhou, China) for 5 min at 2,500 rpm. After that, the eluent was taken out and put into a new tube for cell collection. Fresh PBS was added to the tube for repeated vibration and elution. The subsequent eluent was combined with the previous one and centrifuged at 5,000 g for 10 min to collect the cells. After centrifugation, the supernatant was discarded and the resulting precipitate was resuspended in nutrient bouillon medium and incubated at 37 °C for 24 h. The following day, an appropriate amount of sodium hypochlorite (theoretical value of the final concentration calculated by free chlorine, 9 mg L^{-1}) was added to the eluent (initial screening of chlorine-resistant bacteria). After 2 h of inactivation, when the concentration of free chlorine is guaranteed to exceed 0.5 mg L^{-1}, the microorganisms were isolated using conventional plating methods on R_2A

Table 1 | Physical and chemical parameters for the collected samples ($n = 3$)

Parameters	Value
pH	7.98 ± 0.03
turbidity (NTU)	0.30 ± 0.02
temperature (°C)	26.0 ± 0.1
residual chlorine (mg/L)	0.25 ± 0.02
TOC (mg/L)	2.88 ± 0.07
total dissolved solids (mg/L)	580 ± 6

medium. Plates were incubated aerobically at 22 °C for 7 d for single colonies. Sodium hypochlorite was used as a disinfectant for the screening, isolation, and disinfection of the bacteria. Free chlorine was determined by the spectrophotometric N,N-diethyl-p-phenylenediamine method (Chen *et al.* 2012).

Verification of chlorine resistance for the isolate

The separated colony that had been purified three times was used for chlorine resistance verification. The isolated strain was added to 5 mL of nutrient broth and cultured at 37 °C for 24 h. Bacteria were separated by centrifugation as before (at 5,000 g for 10 min) and washed twice with PBS to remove the remaining culture supernatant. Harvested cells were resuspended in 10 mL of PBS for bacterial suspension preparation. Sodium hypochlorite was added to the suspension for a final dose concentration of 15 mg L^{-1} (calculated by free chlorine, reconfirming the chlorine resistance of the isolated culture). After 2 h of inactivation, sodium thiosulfate was used to terminate the reaction and the R_2A agar plate count method was used to determine the presence of chlorine-resistant bacteria. The resulting single colony was preserved using a HBPT001-1 bacterial bead preservation kit (Qingdao Hi-Tech Industrial Park Hope Bio-technology Co., Ltd, Qingdao, China) for subsequent experiments.

16S rRNA identification

For the identification of 16S rRNA, bacterial cells were grown in R_2A medium at 22 °C for 7 days. Genomic DNA was extracted using a bacterial genomic DNA extraction kit (Biotech, China) according to product instructions. The universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACACTT-3′) were used for 16S rRNA gene amplification (Sun *et al.* 2013). PCR reactions were performed using a thermal cycler (Applied Biosystems, USA) in a total volume of 25 μL. Each reaction consisted of 2.5 μL 10 × Buffer (with Mg^{2+}), 1 μL of 2.5 mM dNTP mix, 0.2 μL DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, USA), 0.5 μL of 10 μM 8F and 1492R primer, 0.5 μL of sample DNA and 19.8 μL of PCR-grade water. The PCR amplification conditions were as follows: an initial activation step at 94 °C for 4 min; and after heating, DNA was amplified for 30 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The products were gel extracted and sequenced with a 3730XL DNA analyzer (Applied Biosystems, USA). Nucleotide sequences were submitted to the BLAST search engine at NCBI GenBank and identified through similarity values (Tamura *et al.* 2007). The 16S rRNA gene sequences from chlorine-resistant bacteria in the previous reports (GenBank accession numbers: AJ536039 and AF480586) were also retrieved from GenBank and used as outgroups. Multiple sequence alignments were carried out by Clustal X program (Thompson *et al.* 1997). A phylogenetic tree was constructed by MEGA software (Molecular Evolutionary Genetics Analysis software, version 5.05), using the neighbor-joining method (Tamura *et al.* 2011) to conduct the bootstrap analysis (1,000 replicates).

Bacterial suspension preparation

Bacterial suspension of JN724 after the exponential phase was prepared as described in section 'verification of chlorine resistance for the isolate'. The isolated strain was added to the nutrient broth and cultured at 37 °C for 24 h. Bacteria were separated by centrifugation as before and washed twice with PBS to remove any remaining culture supernatant. Harvested cells were resuspended in PBS for bacterial suspension preparation. *Escherichia coli* (CICC 10899), obtained from China Center of Industrial Culture Collection (CICC), was chosen as a representative for chlorine-resistance analysis. The culture and bacterial suspension preparation techniques for *E. coli* were the same as those for JN724. In line with previous work (Falkinham *et al.* 2001), cells were resuspended in PBS with an initial cell density of 10^6 CFU mL^{-1} for disinfection treatment.

Chlorine resistance analysis

Chlorine disinfection

To better understand chlorine resistance, *E. coli* was used as a comparison strain in the disinfection experiments. All of

the inactivation tests were conducted in closed glassware, which had been soaked in sodium hypochlorite overnight, rinsed thoroughly, and sterilized by autoclaving. The initial densities of bacteria employed for the disinfection test were 10^6 CFU mL^{-1} in PBS. First, 500 mL of the suspension was added to the glassware. Sodium hypochlorite was then introduced to the bacterial suspension with an equilibrium content of 2.5 mg L^{-1} as free chlorine (for reconfirmation of chlorine resistance). Next, the samples were incubated in a shaking incubator at 25 °C and 100 rpm for 4 h. During the incubation period, the samples were collected from the reactor and counteracted with excess sodium thiosulfate instantaneously at time intervals of 5, 10, 20, 30, 60, 120, and 240 min after inactivation for the HPC, free chlorine analysis and flow cytometry, each in triplicate. The efficacy of disinfectants was determined via assays of the initial and residual concentrations of microbes. Bacteria were estimated following dilution by the plating method (R_2A plates, incubated aerobically at 22 °C for 7 d). Two controls were set up in the experiments: bacteria in PBS without disinfectant and only PBS with disinfectant. The results of three separate trials were used for calculations.

Morphological observation

Observations of morphology were conducted using scanning electron microscopy (SEM) (VEGA3 TESCAN, Brno, Czech Republic). To detect morphological changes in the cell, treated and untreated bacteria suspensions were pelleted by centrifuging as before. After centrifugation, the bacteria were fixed with glutaraldehyde and dried with isoamyl acetate for SEM examination. Sample pretreatments for SEM analysis were performed as described by Wen *et al.* (2017).

FCM analysis

For the flow-cytometric detection, the Cell Viability Kit (BD™, USA) was used to acquire detailed information on the damage to the cell membrane after chlorine treatment. The two nucleic acid stains used were thiazole orange (TO) and propidium iodide (PI), which have different cell permeability properties that can be used to differentiate cells with different membrane integrities. TO, a permeant dye that can enter all cells and bind to nucleic acid, was used to estimate the total number of cells. PI can penetrate the cell membranes of dead cells while being excluded from live cells. The combination of TO and PI provides a reliable method for quantifying cell membrane damage. A 500-μL tested sample was mixed with 5 μL of 42 μmol L^{-1} TO and 4.3 mmol L^{-1} PI prepared in dimethyl sulfoxide (DMSO). It was then incubated in the dark for at least 5 min at room temperature. Liquid counting beads were also added to the tested samples for absolute counting before FCM analysis. Samples were used for FCM analysis (FACSCanto II, BD Biosciences, Franklin Lakes, USA) with 488 nm laser excitation. Data were visualized and obtained from the dot plots of green fluorescence signals versus red fluorescence signals using FACSDiva software (BD Biosciences, Franklin Lakes, USA).

Inactivation experiment

The primary initial cell concentration was about 10^6 CFU mL^{-1} in PBS for both the chlorine dioxide and UV treatments.

Chlorine dioxide

The chlorine dioxide inactivation experiment was performed in a similar manner to the chlorine treatment. First, 500 mL of the prepared suspension was added into the treated glassware. To maintain consistency in the effective chlorine concentration, the primary dose of chlorine dioxide was 1 mg L^{-1}. The glassware was then placed in a shaking incubator at 25 °C and 100 rpm for 30 min. During the incubation period, the samples were collected from the reactor and counteracted with excess sodium thiosulfate instantaneously at time intervals of 5, 10, 20, and 30 min after inactivation for HPC analysis, each in triplicate. The residual chlorine dioxide concentration was determined through spectrophotometric N,N-diethyl-p-phenylenediamine colorimetric analysis (Chen *et al.* 2012). Bacteria in PBS without disinfectant were used as the control in the experiment, and the results were calculated from three separate trials.

UV

The UV irradiation test was carried out using a collimated beam device installed with a low-pressure mercury lamp (R-CANS463RL 40 W, $\lambda = 254$ nm). Irradiance was determined by a radiometer (International Light Technologies, ILT 2400). The intensity of the incident irradiance was approximately 0.168 mW cm^{-2} at the center of the exposure surface. After the lamp was warmed up, a Petri dish was positioned vertically under the collimated beam. A microbial suspension of 40 mL was added to a glass Petri dish reactor, which had a diameter of 60 mm, and the bacterial suspension was stirred. The suspension was irradiated with UV dosages of 5, 10, 15, 20, 40, 80, and 120 mJ/cm^2 at room temperature. Triplicate samples were taken out at scheduled times. The required exposure times were calculated by dividing the desired UV dose by the average UV radiance (Bolton & Linden 2003). The number of viable bacteria was also determined from R_2A agar plates, which had been incubated as described in section 'Chlorine disinfection'. Results were calculated from three separate trials.

Data analysis

The removal efficiency of the disinfection process was expressed as the $\log_{10}$ decrease in bacterial density as per Lin *et al.* (2016):

$$\mathrm{LR} = \log_{10}\left(\frac{\mathrm{N_t}}{\mathrm{N_0}}\right)$$

The disinfection kinetic parameters were obtained by fitting inactivation data to the Chick model (Jensen 2010), formulated as:

$$\log_{10}\left(\frac{\mathrm{N_t}}{\mathrm{N_0}}\right) = -\mathrm{kCT}$$

where LR is the log reduction of bacterial density at time t; N_0 and N_t are the bacterial concentration before and after exposure, respectively; C is the free chlorine concentration in mg/L; T is the contact time in min; and k is the inactivation rate constant in L/(mg•min).

RESULTS AND DISCUSSION

Identification of isolates

The isolates were identified by physiological, biochemical, and molecular biological techniques. Table 2 shows the parameters related to colony morphology, as well as the physiological and biochemical characteristics of JN724. The phylogenetic tree was constructed with the 16S rRNA gene sequences of JN724 and representative sequences of the genus *Gordonia*. The JN724 strain was found to be closely related to both *Gordonia terrae* and *Gordonia hongkongensis*, with which JN724 showed 99% homology (Figure 1). At the same time, the high chlorine resistance of *Gordonia* JN724, which was isolated from the drinking water distribution system, was confirmed. Although *Sphingomonas, Mycobacterium tuberculosis, Legionella pneumophila, Pseudomonas aeruginosa, Staphylococcus aureus*, and other species with high chlorine resistance have been isolated and identified from drinking water systems (Mir *et al.* 1997; Chen *et al.* 2012; Sun *et al.* 2013; Gomes *et al.* 2016), there have been no reports on this highly chlorine-resistant strain of *Gordonia*. In this study, chlorine-resistant JN724 was obtained from the water supply system for the first time. For the screening of

Table 2 | The physicochemical characteristics of *Gordonia* JN724

Category	Parameters	JN724
Colony morphology	Color	Salmon pink
	Form	Round, convex; poor growth
	Diameter	1 mm
	Texture	Smooth and moist
Physiological characteristics	Size	0.5–0.7 × 1.2–2.0 μm; $\varphi \approx 0.7$ μm
	Cell morphology	Short rod; Globular form of young bacteria
	Gram stain	G^+
	Growth temperature	20–45 °C
	Suitable pH for growth	6.5–7.2
Biochemical characteristics	Lactose fermentation	None acid and gas
	Ammonia production test	–
	Oxidase	–
	Catalase	+

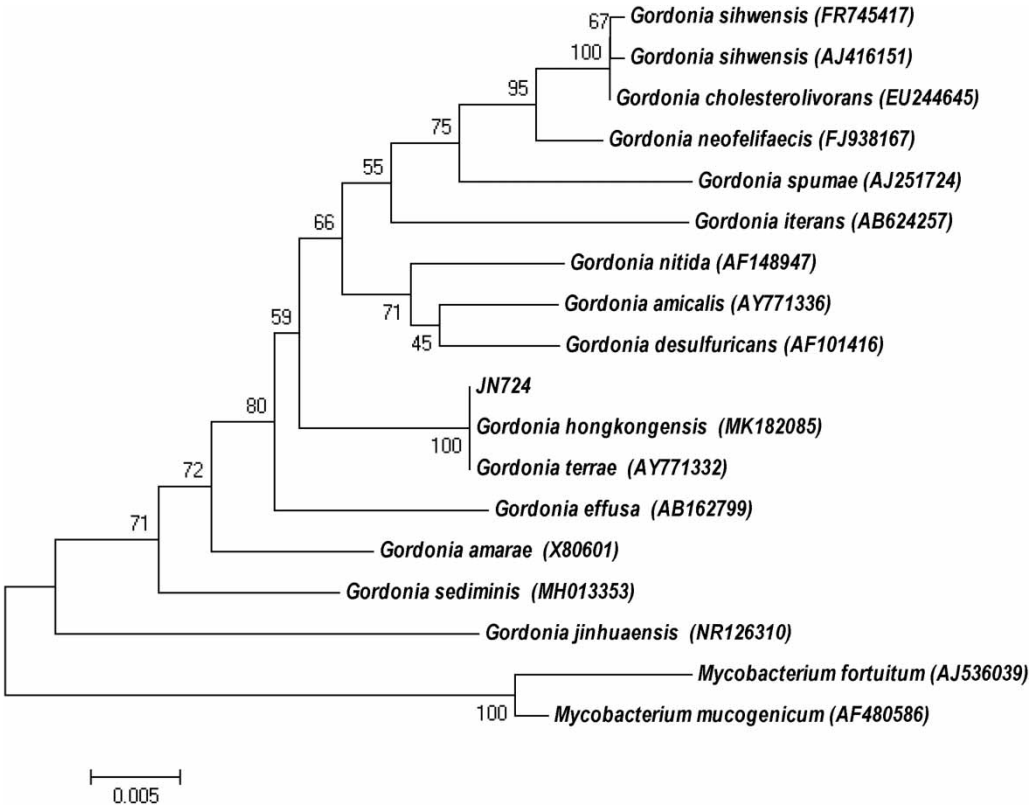


Figure 1 | Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between the JN724 strain and related taxa.

chlorine-resistant bacteria in the local urban water supply system, we found that the occurrence frequency of *Gordonia* was relatively high, although it was not accurately quantified. This is one of the reasons why it was chosen as the object of the study.

It is found that the diversity of isolated bacteria may vary due to different media or isolated technology (Guan *et al.* 2020). In order to minimize the impact of pre-incubation, future research should focus on the two aspects: First, after further enrichment and concentration, the chlorine-resistant strains may be screened by direct chlorination treatment without pre-culture. Second, it is necessary to choose different types of media and culture conditions for meeting the growth needs of more strains in the pre-culture according to previous studies (Schroer *et al.* 2020; Guan *et al.* 2020).

Chlorine disinfection assay

E. coli is a microorganism susceptible to chlorine at the normal dosage required for water disinfection, whereas the isolated strain in this study is known to be chlorine resistant. Consequently, we anticipated a significant difference in chlorine demand and inactivation effects between the two strains. For comparisons of chlorine consumption, disinfectants were mixed into the microbial suspension at an identical initial concentration of 2.5 mg L^{-1} as free chlorine. The primary initial cell concentrations for *Gordonia* JN724 and *E. coli* were 4.0×10^6 CFU mL^{-1} and 3.6×10^6 CFU mL^{-1}, respectively. Free chlorine concentration and chlorine demand were measured and compared. Chlorine demand was expressed as the difference between the concentration of free chlorine before treatment and the amount of chlorine residual at a given exposure time (Helbling & Vanbriesen 2007).

The inactivation kinetics of *Gordonia* JN724 and *E. coli* exposed to chlorine are illustrated in Figure 2. For both species, the concentration of free chlorine decreased as contact time increased. In the first 5 min, the chlorine concentration decreased very quickly, accompanied by a rapid inactivation velocity. Compared with *Gordonia*

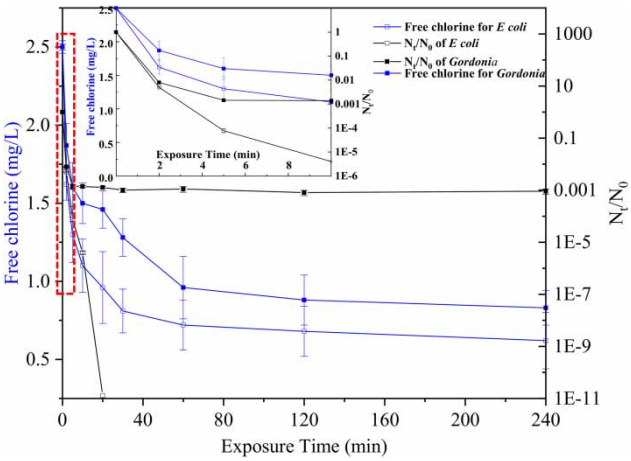


Figure 2 | Free chlorine consumption curves and inactivation efficiency of *E.coli* and *Gordonia* JN724(Nt: the number of *E.coli* and JN724 at different time, CFU/mL; N_0 means the number of *E.coli* and JN724 at time zero, CFU/ ml.

JN724, chlorine consumption rate of *E. coli* was significantly faster and chlorine demand was greater at the end of the experiment (Table 3). In order to determine the effect of organic matter on chlorine consumption, total organic carbons in the supernatants of *Gordonia* JN724 and *E. coli* were measured, and it was shown that there was no significant difference. The faster chlorine consumption rate of *E. coli* indicated that *E. coli* contained more cellular substance that could easily react to the chlorine than *Gordonia* JN724 (Chen *et al.* 2012). In the present study, a higher chlorine demand was observed in the *E. coli* treatment than in previous studies (Zhao *et al.* 2001). The ultimate chlorine demand of free chlorine exceeded 1 mg/L for 99.99% inactivation. It was previously reported that chlorine consumption would increase with the growing initial dosage of chlorine disinfection in *E. coli* treatment (Helbling & Vanbriesen 2007). The free chlorine concentration used in the present study was much higher than those in other studies, and that may have been a reason for the observed high chlorine consumption. Despite the continuous free chlorine consumption, there was no significant progress in the *Gordonia* JN724 inactivation. The subsequent depletion of residual chlorine may be interpreted as a secondary response to the substances released in the dead cells. Additionally, the concentration of free chlorine decreased when HClO was added to the sterilized PBS buffer solution (Wang *et al.* 2019). Therefore, more detailed investigation is needed to explore the continued consumption of free chlorine.

Table 3 | The chlorine demand of *Gordonia* JN724 and *E.coli* in chlorine inactivation ($n = 3$)

	Chlorine demand (mg/L)	
Reaction time (min)	***E. coli***	**JN724**
5	1.20 ± 0.07	0.9 ± 0.10
10	1.40 ± 0.09	1.00 ± 0.11
20	1.54 ± 0.09	1.04 ± 0.09
30	1.69 ± 0.15	1.22 ± 0.07
60	1.78 ± 0.05	1.54 ± 0.10
120	1.82 ± 0.08	1.62 ± 0.08
240	1.88 ± 0.08	1.67 ± 0.09

Reduction of *E. coli* ranged from 6 log units to 1 log unit after 10 min of exposure (LR was about −5), and no viable count was detected in the plate after 20 min of exposure. *E. coli* was considered to have been entirely inactivated by chlorine in the traditional sense. Within the first 5 min (LR was about −3), chlorine eliminated about 3 log units of *Gordonia* JN724. The number of viable bacteria remained relatively constant from 5 min to the end of the experiment (LR was about −3), and the final determination of *Gordonia* JN724 in the plate was above 3 log units (for a contact time of 240 min). There was a clear demarcation point at 5 min in the survival curves, as an initial speedy inactivation period before and a slow or an ineffective inactivation stage after. The result is in accordance with many previous studies on the two stages in the response curve (Luh & Mariñas 2007; Chen *et al.* 2012). These two stages may be attributed to the existence of two populations – one consisting of susceptible cells and the other of tolerant cells. One possible explanation for the presence of two populations may be that the cultures contained multiple colony variants. The existence of colony variants and the spontaneous transition between colony types have been reported in the past (McCarthy 1970; Woodley & David 1976; Stormer & Falkinham 1989). Similar to that reported for free chlorine, the two-population analysis appeared following chlorine dioxide and ultraviolet radiation treatment. The idea that a 'weak' group and a 'strong' group exist in disinfection experiments has also been proposed (Benito *et al.* 2002; Vicuna-Reyes *et al.* 2008).

Morphological observations

SEM photos of *Gordonia* JN724 and *E. coli* before and after chlorine inactivation are shown in Figure 3. Prior to treatment, both were rod-shaped and their surfaces were glossy. Following inactivation, the surface of *E. coli* became incomplete, and holes appeared on the surface of the membrane. With increased contact time, the cell membrane of *E. coli* deteriorated, and the complete cell morphology could not be observed in the later experiment. However, no major changes in membrane integrity were seen in *Gordonia* JN724; only its shape changed. This difference in morphology further confirmed that *Gordonia* JN724 is much more resistant to chlorine than *E. coli*.

FCM analysis

The stain combination of TO and PI was used to evaluate the permeability of the cell membrane and thus distinguish between live cells (with an intact membrane) and dead cells (with an injured membrane) after inactivation. According to the distinction in fluorescence species and intensity, the stained individuals are represented in different areas of the dot plot. There was adequate separation between the background noise and the stained individuals (Hammes & Egli 2010). The gate applied for the quantification of the total cell concentration was labeled '*E. coli*' or '*Gordonia* JN724' and another was 'beads' in FCM dot plots (FSC and SSC). According to the distinctions in the TO and PI stains, the quantification region was divided into three regions: 'live', 'injured', and 'dead'. Live (bacteria which were cultured in nutrient broth at 37 °C for 24 h) and completely dead cells (heat-killed, 100 °C hot water for 10 min) were used as controls to designate the shapes of the 'live' and 'dead' regions. The 'injured' region was obtained by cells from the fluorescence mixture of the 'live' and 'dead' regions (Zhang *et al.* 2018). Cells in the 'injured' region consisted of VBNC individuals that could not be detected by culture methods.

Cells stained before and after the inactivation are shown in Figures 4 and 5. The data provide support for the use of TO and PI double staining associated with flow cytometry as a cultivation-independent approach for evaluating both total cell concentrations and cells with damaged membranes in *E. coli* inactivation. Before the inactivation, the

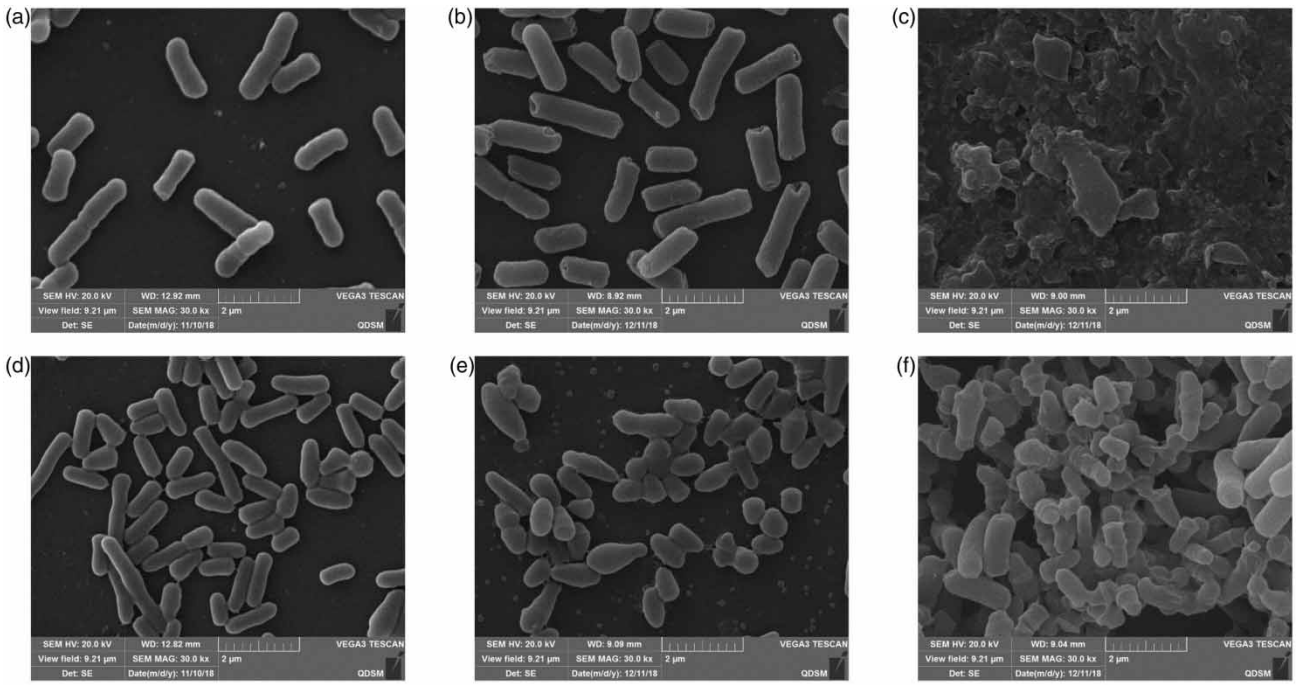


Figure 3 | SEM photos of *E. coli* and *Gordonia* JN724 before and after chlorine inactivation: (a) *E. coli* before inactivation; (b) *E. coli* after 5 min inactivation; (c) *E. coli* after 2 h inactivation; (d) JN724 before inactivation; (e) JN724 after 5 min inactivation; (f) JN724 after 2 h inactivation.

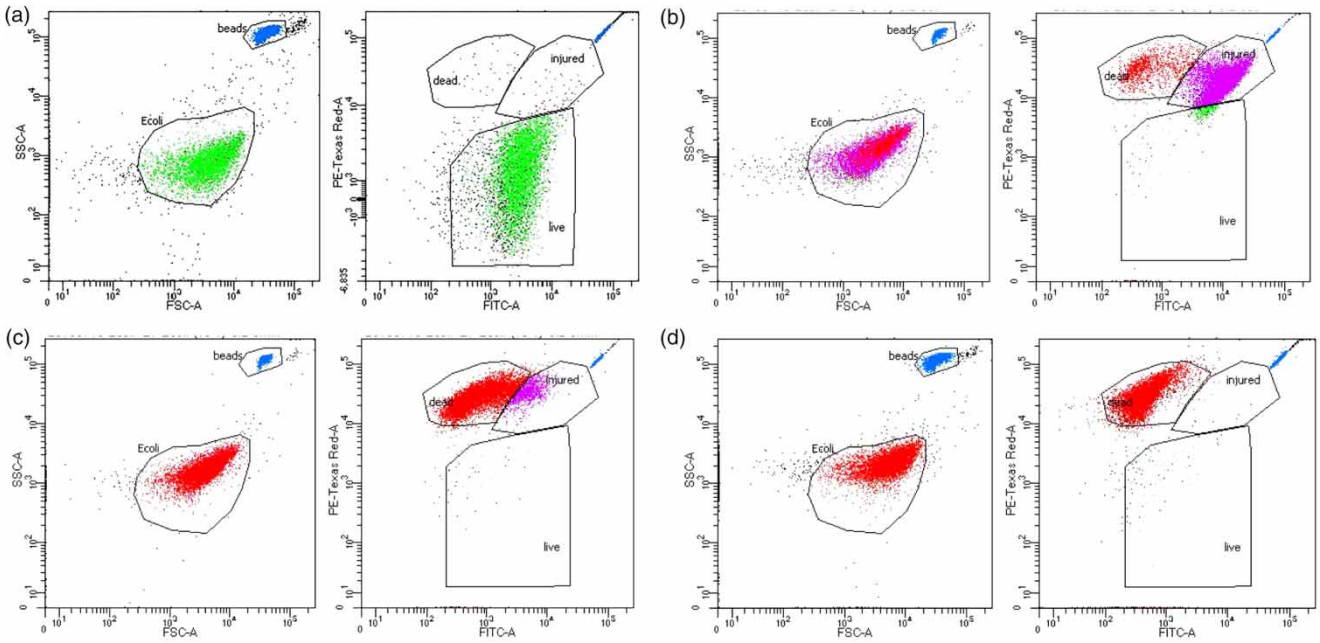


Figure 4 | Representative FCM dot plots of *E. coli* stained with TO and PI to assess membrane integrity: (a) before inactivation; (b) after chlorine exposure for 5 min; (c) after chlorine exposure for 20 min; (d) after chlorine exposure for 2 h. 'FSC-A' means forward scatter and 'SSC-A' means side scatter; 'FITC-A' represents green fluorescence channel and 'PE-Texas Red-A' represents red fluorescence channel; 'live' (green), 'injured' (purple) and 'dead' (red), which corresponded to viable and culturable cells, VBNC cells and dead cells, respectively. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2020.143.

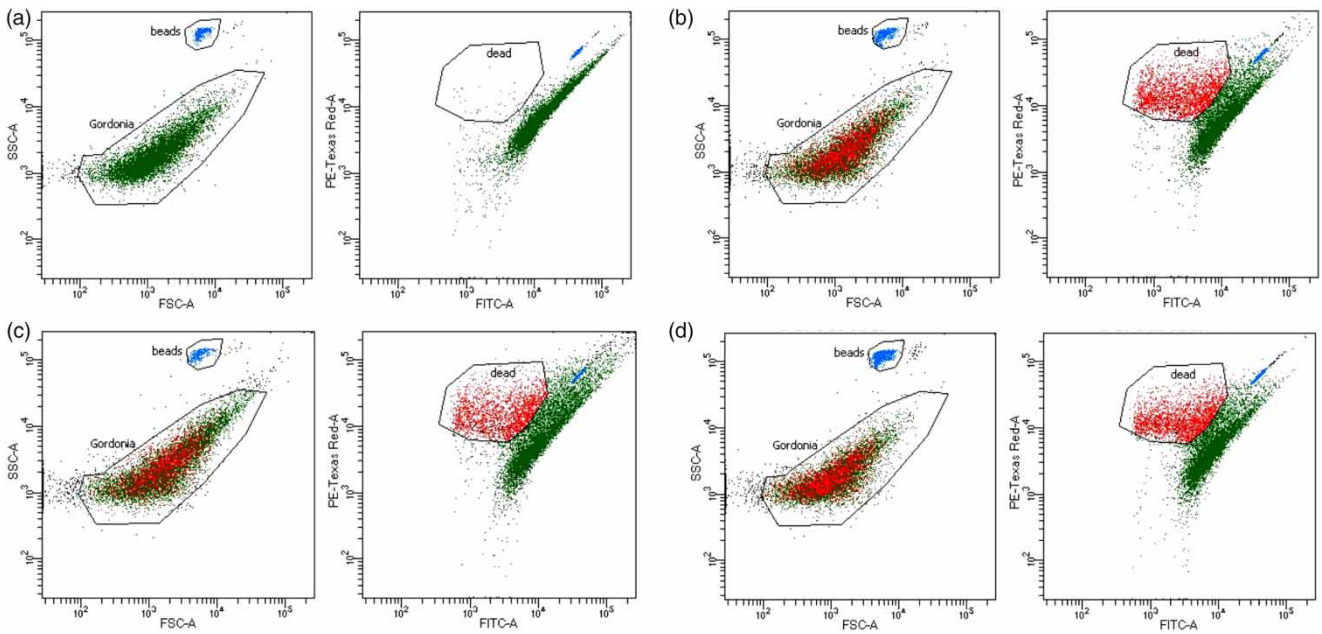


Figure 5 | Representative FCM dot plots of *Gordonia* JN724 stained with TO and PI to assess membrane integrity: (a) before inactivation; (b) after chlorine exposure for 20 min; (c) after chlorine exposure for 2 h; (d) after chlorine exposure for 4 h. 'FSC-A' means forward scatter and 'SSC-A' means side scatter; 'FITC-A' represents green fluorescence channel and 'PE-Texas Red-A' represents red fluorescence channel; 'dead' (red), which corresponded to dead cells. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2020.143.

proportion of 'live' cells was over 98%, whereas the sum of 'injured' and 'dead' cells was less than 2%. However, there was a marked increase in the quantity of 'injured' and 'dead' bacteria with prolonged contact time. The percentage of cells in the 'injured' and 'dead' regions rose to 81.85% and 14.4%, respectively, after 5 min of chlorine treatment. After 20 min of exposure, only about 20% of the cells appeared in the 'injured' region (VBNC state), and no viable counts were detected by traditional methods. All of the cells were 'dead' within 2 h. Bacteria entered a VBNC state in the chlorine treatment that was not detectable by the cultivation method.

In the present assay, abnormal cell membrane permeability for *Gordonia* JN724 was observed. Only total cell concentrations and 'dead' cells were measured, while cells with damaged membranes were not detected. However, we discovered that normal microbial cells could be stained by TO and PI at the same time, with all of the cells located in the 'injured' region. Red fluorescence can be observed in living cells with a combination of PI and nucleic acid in the repeated test. Thus, it is impossible to identify the real state of cells using FCM, as 'live' and 'injured' appear in the same region. The number of cells in the 'dead' region increased with exposure time. From 20 min exposure to the end of the treatment, the proportion of dead cells remained relatively stable (20–30%), which also confirmed the chlorine resistance of *Gordonia* JN724 on the other sides. For VBNC cell detection, it may be possible to try other types of dyes in future.

FCM analysis has been used to detect bacterial activity in water disinfection, and more challenges have been uncovered, such as the existence of VBNC cells (Nie *et al.* 2016; Zhang *et al.* 2018). A past study found that while pathogens in the VBNC state could not trigger disease directly, potential virulence was retained, and they were able to initiate infections with recovery to a metabolic state (Zhang *et al.* 2018). Our study also confirmed the existence of a large number of VBNC cells following chlorine disinfection. The fraction of VBNC cells decreased with increases in available chlorine and contact time. Because of the potential to cause harm later, the VBNC state must be examined in further detail.

Previous studies showed that the disinfectant resistance of bacteria may be attributed to differences in cytoarchitecture, components of the cell wall, cell size, and other aspects (Sisti *et al.* 2012; Wen *et al.* 2016). FCM analysis revealed that the number of cells that PI completely penetrated was only less than 30% for *Gordonia* after 30 min of chlorine exposure, while that of *E. coli* was more than 80%. The difference in membrane integrity and permeability between the two strains were likely responsible for the difference in chlorine tolerance and are consistent with the SEM observations. Gram-positive bacteria are generally more resistant to gram-negative bacteria because their thicker cell walls can withstand the oxidation effects of hypochlorous acid and hypochlorite (Nie *et al.* 2016). This phenomenon may explain the strong resistance of the cytomembrane of *Gordonia* JN724 to chlorine. It remains unclear whether the special blocking structure of the bacteria lead to TO and PI double staining and contribute to its chlorine tolerance. To learn more about the resistance mechanisms expressed in *Gordonia* JN724, further research on its surface structure is needed, including the protein and fatty acid composition, as well as intracellular interactions.

Inactivation experiment

With 0.5 mg L^{-1} and 1 mg L^{-1} chlorine dioxide exposure, no colonies were detected by the HPC method after a short time exposure. Subsequently, 0.38 mg L^{-1} of chlorine dioxide treatment was performed. *Gordonia* JN724 exposed to 0.38 mg/L of chlorine dioxide for 5 min produced more than 2 log inactivation (Figure 6). The CT value for 99.9%

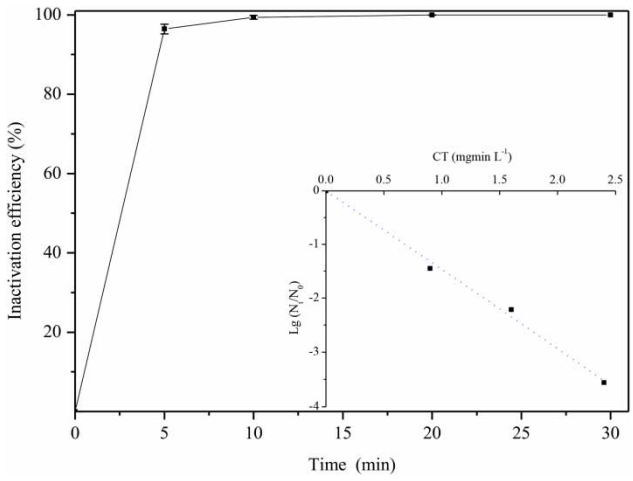


Figure 6 | Dose response of *Gordonia* JN724 after UV irradiation (Nt: the number of JN724 at different time, CFU/mL; N_0 means the number of JN724 at time zero, CFU/mL).

inactivation was calculated as 2.04 mg min L^{-1}, which was notably lower than that of free chlorine. This suggests that *Gordonia* JN724 is more sensitive to chlorine dioxide than chlorine.

Many studies have found chlorine dioxide to be more effective than free chlorine in the inactivation of bacteria (Chen *et al.* 2012; Wen *et al.* 2017). The oxidation potential if chlorine dioxide oxidation potential (redox potential: 435 1.51 V) is higher than that of chlorine (redox potential: 1.36 V) (Hosni *et al.* 2013), which will lead to a more rapid reaction with intracellular materials. Additionally, chlorine dioxide, a neutral molecule in water, can easily diffuse through the cell wall membrane (Gagnon *et al.* 2004). The higher oxidation potential and permeabilization of chlorine dioxide will thus result in more efficient inactivation compared with chlorine.

The UV dose-response curve in Figure 7 indicated that UV irradiation was effective in *Gordonia* JN724 inactivation. When the UV dosage increased to 40 mJ cm^{-2}, approximately 4 log 10 (i.e., 99.99%) removal was detected. Complete inactivation (about 5 log 10) was practically reached after exposure to UV fluences of 80 mJ cm^{-2}. The UV dose-response curve for *Gordonia* JN724, which was similar to that of other organisms inactivated by UV (Sun *et al.* 2013), converged with the polynomial regression model (USEPA 2006).

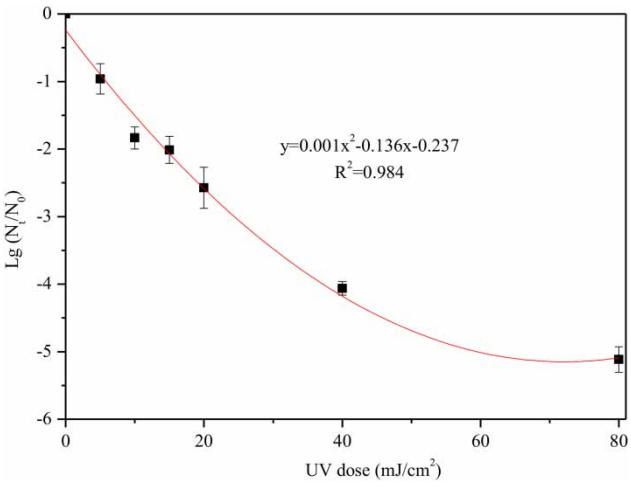


Figure 7 | Dose response of *Gordonia* JN724 after UV irradiation (Nt: the number of JN724 at different time, CFU/mL; N_0 means the number of JN724 at time zero, CFU/mL).

According to the inactivation experiment, chlorine dioxide and UV irradiation are fast and effective disinfection methods for *Gordonia* JN724 in water. Many studies have shown that a portion of chlorine-resistant microbes are sensitive to UV irradiation, chlorine dioxide inactivation, and other disinfection methods (Linden *et al.* 2002; Drescher *et al.* 2011; Sun *et al.* 2013). Similarly, *Gordonia* JN724, which was isolated from a water-pipe system in this study, showed a high sensitivity to UV irradiation. The inactivation rate reached 99.99% at a dose of 40 mJ cm^{-2}, which is suggested by the USEPA as the optimal density in drinking water treatment (USEPA 2006). The CT value for 99.9% inactivation by chlorine dioxide was approximately 20 times lower than that of chlorine disinfection. Therefore, chlorine dioxide or UV disinfection may be alternative approaches for the control of chlorine-resistant microorganisms.

CONCLUSIONS

Gordonia JN724 is a highly chlorine-resistant, gram-positive bacterium obtained from the drinking water distribution system taking Yellow River as the water source. FCM analysis confirmed the presence of a great majority of VBNC cells and damage to cell membranes in *E. coli* inactivation. The structural properties of chlorine-resistant bacteria must be studied systematically for chlorine-resistant mechanisms. *Gordonia* JN724 can be effectively inactivated by ClO_2 and UV treatments, which suggests that they may serve as alternative approaches for the control of chlorine-resistant microorganisms. Meanwhile, water treatment plants using chlorine disinfection should be aware of the existence of chlorine-resistant microbes and consider different disinfection methods based on their own unique needs.

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CONFLICT OF INTEREST

No conflict of interest declared.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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