Original Article

Inactivation of Resistant *Mycobacteria mucogenicum* in Water: Chlorine Resistance and Mechanism Analysis*

CHEN Yu Qiao, CHEN Chao#, ZHANG Xiao Jian, ZHENG Qi, and LIU Yuan Yuan

School of Environment, Tsinghua University, Beijing 100084, China

Abstract

Objective To better understand the mechanism of chlorine resistance of mycobacteria and evaluate the efficiency of various disinfection processes.

Methods Inactivation experiments of one strain *Mycobacteria mucogenicum,* isolated from a drinking water distribution system in South China were conducted with various chlorine disinfectants. Inactivation efficiency and disinfectant residual, as well as the formation of organic chloramines, were measured during the experiments.

Results This strain of *M. mucogenicum* showed high resistance to chlorine. The CT values of 99.9% inactivation by free chlorine, monochloramine and chlorine dioxide were detected as 29.6±1.46, 170±6.16, and 10.9±1.55 min•(mg/L) respectively, indicating that chlorine dioxide exhibited significantly higher efficiency than free chlorine and monochloramine. It was also found that *M. mucogenicum* reacted with chlorine disinfectants more slowly than *S. aureus,* but consumed more chlorine disinfectants during longer time of contact. Lipid analysis of the cell construction revealed that 95.7% of cell membrane lipid of *M. mucogenicum* was composed of saturated long chain fatty acids. Saturated fatty acids were regarded as more stable and more hydrophilic which enabled the cell membrane to prevent the diffusion of chlorine.

Conclusion It was concluded that different compositions of cell membrane might endow *M. mucogenicum* with a higher chlorine resistance.

Key words: Chlorine inactivation; Chlorine consumption; Lipid assay; Mechanism; *M. mucogenicum*

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INTRODUCTION

Recently the emerging resistant mycobacteria in the water distribution system have attracted an ever increasing attention in the field of drinking water treatment and public health[1-3]. The existence of mycobacteria in China has also been reported in recent years[4-6]. Mycobacteria are considered opportunistic pathogens that can cause infection or disease in individuals with immunocompromised conditions[7-8]. And it has also been reported that *M. avium* causes progressive parenchymal lung disease and bronchiectasis in persons without predisposing conditions[9]. Many available references on mycobacteria control have studied the relationship between the growth of mycobacteria and water quality parameters, such as temperature, pH, AOC level, residual disinfectants, and others[10-16]. However, temperature is fairly stable in the underground distribution system; the updating of water treatment process to improve pH, AOC levels are not easy to be applied in the

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#Correspondence should be addressed to CHEN Chao. Tel: 86-10-62781779. Fax: 86-10-62781779. E-mail: chen_water@tsinghua.edu.cn

Biographical note of the first author: CHEN Yu Qiao, female, born in 1986, master, majoring in civil engineering.

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developing countries, including China. Therefore, disinfection is the most practical way to control the growth of mycobacteria in the distribution system. Chlorine disinfection processes is most widely used in water treatment plants in China because it is cost effective and could keep active in the distribution system for a considerable length of time[17]. Alternative disinfectants, such as chloramines and chlorine dioxide have also been widely applied in China in recent years.

A number of mycobacteria have been shown to be relatively resistant to chlorine disinfectants at concentrations used in the water distribution system[10-11,14,18]. Unfortunately, most researches were conducted by using free chlorine and no comprehensive study has been reported on other chlorine disinfectants such as monochloramine and chlorine dioxide. The research of chlorine disinfection mechanism is relatively behind the disinfection practice which limits the improvement of disinfection methods. Therefore, it is necessary to evaluate the efficiency of various disinfection processes and to better understand their principles.

At the same time, the mechanism for the survival of mycobacteria in drinking water is not well understood. Cloete's study showed that the resistance of bacteria to free chlorine was attributed to unique variations in the cellular structure and protein composition[19]. Some researchers studied the chlorine consumption of various bacteria and argued that the special cell composition of mycobacteria consumed more disinfectants. Shang and Blatchley[20] reported that bacteria reacted with free chlorine disinfectants rapidly and formed the organic chloramines which were generally recognized as poor antimicrobial agents[21]. Further studies on chlorine demand concluded that different organisms had unique chlorine demands, even under the condition of similar organic carbon content and at equivalent initial cell concentrations. Helbling's researches have shown that *M. aurum* consumed much more chlorine than *Staphylococcus epidermidis* and *Escherichia coli*[22].

In this study, experiments of mycobacteria inactivation with various chlorine disinfectants were conducted. Inactivation efficiency and disinfectant residual, as well as the formation of organic chloramines, were measured during the experiments. Mycobacterium was compared with a relative chlorine sensitive bacterium, *Staphylococcus aureus* in the disinfection and lipid analysis experiment. An integrated study of the characteristic of disinfectant resistance, including the chlorine demand, bacteria survival and cell structure composition, would help to understand how various chlorine disinfectants react with bacteria and why mycobacteria have higher resistance to chlorine. The finding of this study could provide information needed for the control of chlorine resistant bacteria in the water distribution system.

MATERIALS AND METHODS

Bacterial Suspension Preparation

One strain of bacteria was isolated from a water distribution system in South China. This strain was identified as *M. mucogenicum* by 16sRNA and *ropB* gene sequences analysis using current techniques by China Industrial Culture Collection (CICC). *Staphylococcus aureus* (ATCC 6538) was selected as a reference strain on the basis of their relevance to drinking water distribution system and their fairly higher chlorine resistance compared with *E. coli*.

After the exponential phase, cells were harvested by centrifugation at 5 000 rpm for 10 min and washing in an equal volume of 0.05 mol/L chlorine demand-free phosphate buffer solution (PBS) at pH=7. This operation was repeated three times to remove the residual media. The dissolved organic carbon (DOC) (shimadzu, Japan) and ammonia in the solution were monitored[23] to ascertain absence of obvious interference caused by the residual media. PBS was prepared by mixing 420 mL of 0.05 mol/L KH_2PO_4 with 580 mL of 0.05 mol/L Na_2HPO_4. Cells were resuspended again in the same PBS to make the bulk solution with the concentration of 10^6-10^7 CFU/mL[11].

Cell Survival assay During the Disinfection Experiments

All the disinfection experiments were conducted under the condition of chlorine-demand free glassware, pH=7.0 with PBS and 20 °C with gentle shaking in water bath. For each flask, 2 mL of bulk solution were diluted at 1:100 by 198 mL PBS. Free chlorine, chloramines or chlorine dioxide at the same dosage of 1 mg/L (as available chlorine) was added to the polluted water respectively. After 0, 5, 10, 20, 30, 60, 120, 240, and 1 440 min of inactivation, samples were taken out of the reactor and quenched with 100 μL of sterile of 0.1 mmol/L sodium thiosulfate. The initial and residual concentrations of microbes were assayed to determine the efficacy of disinfectants. The detection of *M. mucogenicum* was performed with R2A nutrient agar plate count at

22 °C for 7 days. *S. aureus* was cultured with broth nutrient agar plate count at 37 °C for 24 h[24]. Each point of data was obtained by three dilution ratios and a blank control.

Chlorine Disinfectants Preparation and Determination

Free chlorine and chlorine dioxide solutions were prepared by diluting concentrated stock solution. Monochloramine stock solutions were prepared daily at a concentration of 400 mg/L as Cl_2 by mixing sodium hypochlorite and ammonium sulfate solutions at a weight ratio of Cl_2:N=4:1[25]. Both solutions were pre-adjusted to pH=9. At this pH, monochloramine was the dominant species of inorganic chloramines; neither dichloramine nor trichloramine was detected. All glassware used for disinfection studies was critically cleaned, rinsed with deionized, glass-distilled water and heated in a dry-air oven.

Free chlorine and total chlorine were measured by the DPD N,N-diethyl-p-phenylenediamine colorimetric method (MDL=0.02 mg/L as Cl_2) using the free chlorine and total chlorine powder reagents and pocket colorimetric (Hach Co.,USA). Monochloramine was measured using monochlorF reagent (Hach Co.,USA) based on indophenols method which had been verified by using the membrane introduction mass spectrometric system that it only detected inorganic monochloramine, instead of organic chloramines[17]. Organic chloramines concentration was calculated by subtracting free chlorine and monochloramine concentrations from the total chlorine concentration. It was verified that nitrogen had been washed away by the pretreatment mentioned before[23]. Meanwhile, under the experimental conditions, dichloramine and trichloramine were not detected.

Ct Value Determination

Linear regressions based on the logarithm of the percent survival for each strain of the bacteria were calculated and expressed as Ct value, the product of disinfectants concentration (in mg/L) and time (in minute). As reacting with organic compounds, the concentration of disinfectants decreased during the experiments. In order to accurately evaluate *Ct* values, chlorine decay was integrated as a function of time: (as described in[26])

$$\log_{10}(\frac{N_0}{N}) = -k_i \int_0^t Cdt$$

Lipid Assay

M. mucogenicum and *S. aureus* were compared for the difference of their lipid composition. This work was conducted by the Institute of Microbiology, Chinese Academy of Science. Fatty acids were analyzed by the method described by Stewart and Olson[27]. Briefly, fatty acids were released from the cells by the addition of 1.0 mL of 15% NaOH in 50% methanol with heating for 30 min in a boiling water bath. The fatty acids were methylated by addition of 2 mL of 3 mol/L HCl in 50% methanol in an 80 °C water bath. The aqueous extract was then washed with 1.25 mL of hexane-ether (1:1, vol/vol) by tumbling on a hemocytology mixer for 10 min. Finally, the aqueous wash of the organic phase was performed by addition of 3 mL of 1.2% NaOH and tumbling for 5 min. The organic phase was then analyzed with a gas chromatograph (MIDI Microbial identification) and automatic bacteria identification system (Sherolock Co.). Replicate analyses were conducted to confirm the accuracy of the results.

RESULTS

Inactivation of M. mucogenicum by Three Chlorine Disinfectants

Three kinds of disinfectants, i.e. free chlorine, monochloramine and chlorine dioxide, performed obviously different inactivation modes when inactivating *M. mucogenicum* with the same addition dosage. Chlorine dioxide had significant higher efficiency than other two kinds of chlorine disinfectants and eliminated 5 log units of mycobacteria within 60 min. This result was in agreement with Taylor's research of chlorine susceptibility of *M. avium* that chlorine dioxide had better inactivation efficiency than free chlorine[28].

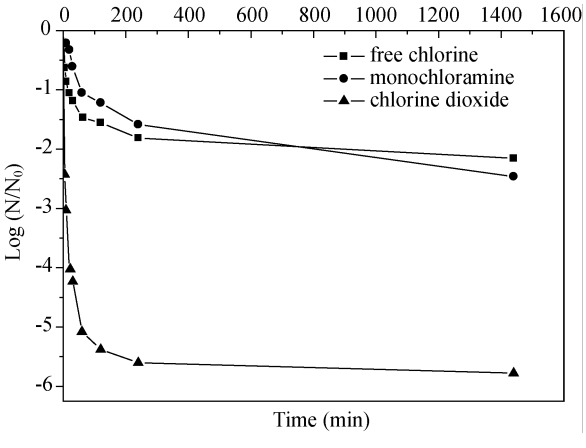

Figure 1. *M. mucogenicum* survival curve with three kinds of chlorine disinfectants. *Note*: Dosage of all chlorine disinfectants were 1 mg/L as Cl_2. The initial bacteria concentration= 6.1×10^6 CFU/mL.

The survival curves could be divided in two stages: an initial fast inactivation stage and a slow and tailing inactivation period. This result was consistent with Luh's study about the inactivation of *Mycobacterium avium*[14,29]. Chlorine dioxide had the fastest inactivation rate in the first 30 min. Free chlorine inactivated mycobacteria more quickly within 30 min than monochloramine. As the contact time increased, the inactivation curve of free chlorine turned to level off, whereas monochloramine maintained a relatively stable residual concentration and achieved better inactivation efficiency than free chlorine during 24 hours' experiments.

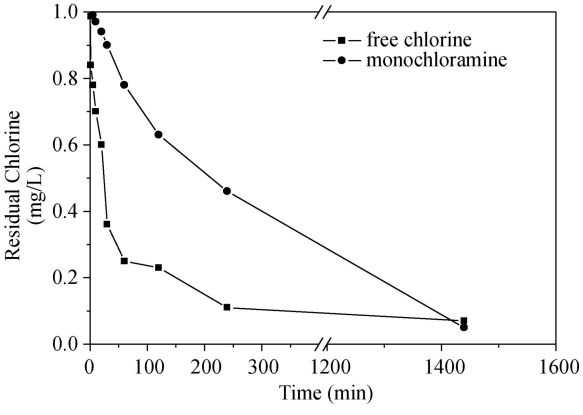

Figure 2. Curves of different chlorine disinfectants decay during the *M. mucogenicum* inactivation experiments. *Note.* Free chlorine, monochloramine was added with the same initial concentration of 1 mg/L as Cl_2. Initial number of bacteria, $N_0=6.1\times10^6$ CFU/mL.

Disinfectants decay partly explained the difference of inactivation modes between free chlorine and monochloramine. It has been proved that there are significantly different dynamic decay characteristics of free chlorine and monochloramine during disinfection process[30]. Lee[17] observed that chlorination rapidly formed organic chloramines when reacted with natural organic matters, while chloramination formed organic chloramines by 6-720 times slower than free chlorine. Moreover, free chlorine formed larger amount of organic chloramine than monochloramine.

According to Figure 2, residual free chlorine decreased to lower than 0.2 mg/L after 120 min and was close to 0.1 mg/L after 240 min while residual monochloramine was as high as more than 0.6 mg/L after 120 min. Free chlorine also formed more organic chloramines (0.25 mg/L within 2 min) which had no inactivation efficacy. Monochloramine decayed slowly and formed limited concentration of organic chloramines. The concentration of organic chloramines reached its peak of 0.03 mg/L at 2 min and decreased to 0.02 mg/L at 120 min. When combining the bacteria survival curves with Figure 2, it clearly showed that chlorine decay and formation of organic chloramines influenced the inactivation efficiency of *M. mucogenicum*.

Ct Value Determination

The Ct values of 99.9% inactivation of *M. mucogenicum* and *S. aureus* by three chlorine disinfectants were calculated in this study, as illustrated in Figure 3. The Ct values of 99.9% inactivation of *M. mucogenicum* were measured as 29.6±1.46, 170±6.16, 10.9±1.55 min•(mg/L) for free chlorine, monochloramine and chlorine dioxide respectively based on triplicate measurements. Those of *S. aureus* were 0.46±0.51, 13.08±0.36, and 0.37±0.84 min•(mg/L). The results showed that *M. mucogenicum* was 20-50 times more resistant to chlorine disinfectants (including free chlorine, monochloramine, chlorine dioxide) than *S. aureus*.

Chlorine Consumption

Chlorine consumption was monitored when two strains of bacteria were inactivated by three kinds of chlorine disinfectants. Figure 4 showed the comparison of free chlorine decay and organic chloramines formation during the inactivation of *M. mucogenicum* and *S. aureus*.

S. aureus had significantly faster chlorine consumption rate than mycobacteria, indicating that it contained more cellular matters easily to react with chlorine such as enzyme upon surface. Moreover, *S. aureus* consumed 0.49 mg/L more free chlorine than *M. mucogenicum* at 10 min while organic chloramines formation of *S. aureus* was just 0.17 mg/L more than that of *M. mucogenicum*. It demonstrated that more effective chlorine got into *S. aureus* and inactivated the bacteria cells. Meanwhile for *M. mucogenicum*, there were no special organic materials consuming chlorine disinfectants according to its relatively slow reaction rate. So it could be speculated that chlorine resistance of mycobacteria with its special cellular compounds was due to its barrier preventing diffusion rather than consuming disinfectants by reaction.

To compare the chlorine consumption, three disinfectants were added at the same initial concentration of 1.0 mg/L as Cl_2. The primary initial

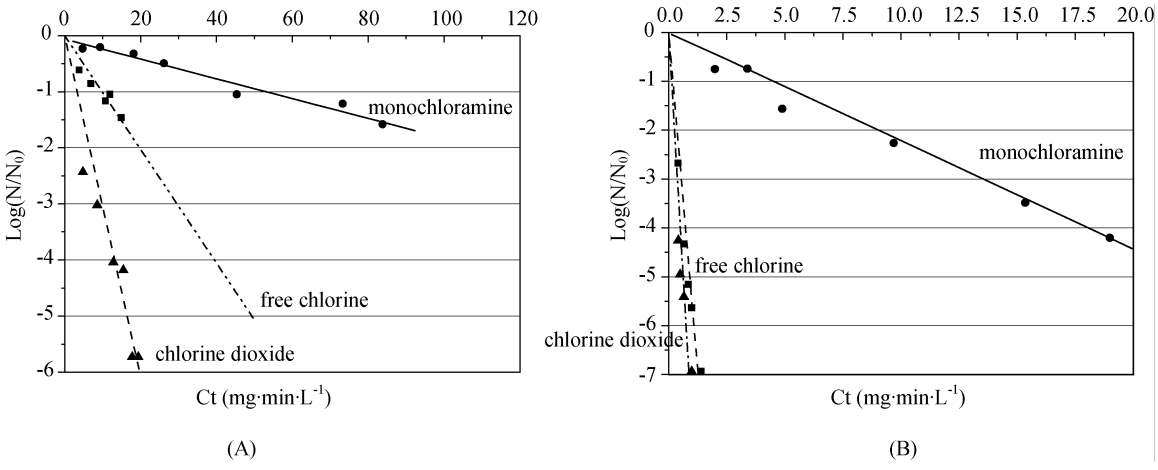

Figure 3. (A) Inactivation of *M. mucogenicum* with three chlorine disinfectants. (B) Inactivation of *S. aureus* with three chlorine disinfectants (i.e. free chlorine, monochloramine and chlorine dioxide). *Note*. pH=7, T=20 °C, Initial chlorine concentration of 1.0 mg/L (as Cl_2). Initial number of *M. mucogenicum* =6.1×10^6 CFU/mL, Initial number of *S.aureus* =8.7×10^6 CFU/mL.

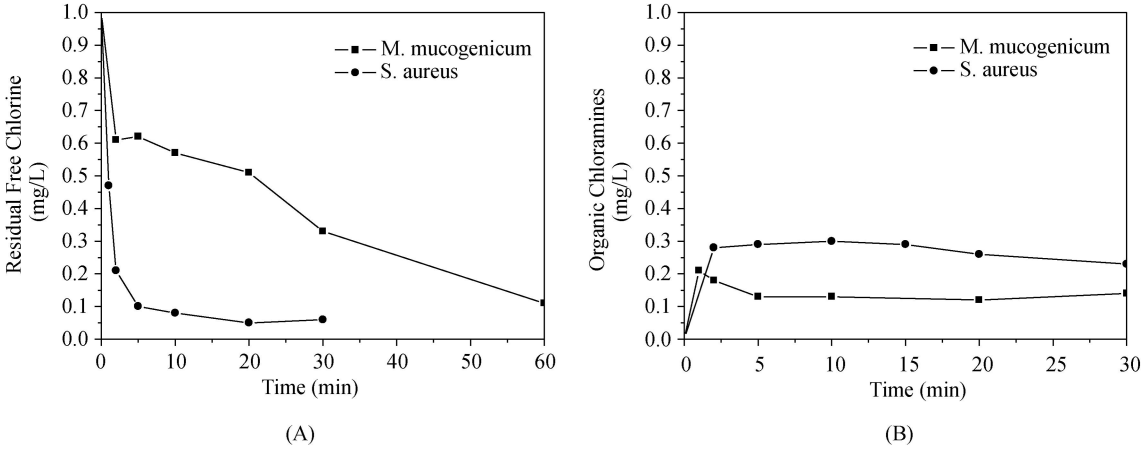

Figure 4. (A) Free chlorine decreased with time during the inactivation experiments of *S. aureus* and *M. mucogenicum*. (B) Formation of organic chloramines during the inactivation process of *S. aureus* and *M. mucogenicum*. *Note*. Initial concentration of *M. mucogenicum* and *S. aureus* were 1.2×10^7 CFU/mL and 1.4×10^7 CFU/mL respectively.

cell concentration for *M. mucogenicum* and *S. aureus* were 1.2×10^7 CFU/mL and 1.4×10^7 CFU/mL respectively. The normalized consumptions of disinfectants were calculated by dividing the total consumption by the inactivated cell number with the unit of mg/L·10^7 CFU in a long enough contact time, as summarized in Figure 5. The self decay of disinfectants was subtracted from the total consumption by blank control without bacteria.

Although the reactions between mycobacteria cell and chlorine disinfectant were slower, the final disinfectant consumptions of mycobacteria were higher than those of *S. aureus*. Similar result was presented in the reference[22]. The ultimate chlorine demand of free chlorine for mycobacteria was approximately 65% higher than that of *S. aureus.* The difference between two strains of bacteria with monochloramine was even more significant. *M. mucogenicum* consumed monochloramine 7 times as high as *S. aureus* when the same amount of bacteria was inactivated. However, no significant difference of chlorine dioxide consumption was observed between the two strains of bacteria.

The results of organic chloramines formation indicated that the reaction between bacteria and disinfectants was much quicker than the bacteria

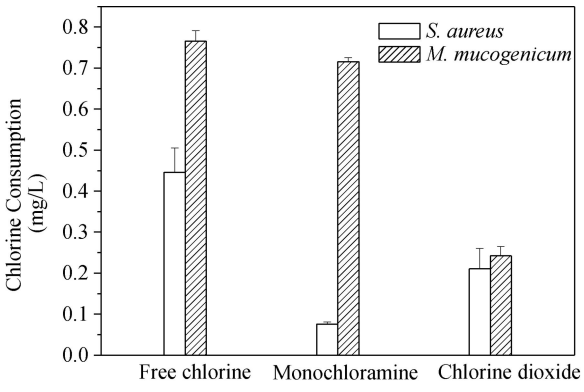

Figure 5. Comparison of normalized chlorine consumption when inactivating 10^7 CFU of bacteria cells by three different disinfectants. *Note:* The normalized chlorine consumption is defined as the dividing the difference between the initial and final chlorine residual with the activated bacteria number of 10^7 CFU. The normalized chlorine consumption was obtained in the unit of mg/L·10^7 CFU.

death. It was interesting that the modes of organic chloramines formation and the inactivation of the two strains of bacteria were quite different. Free chlorine inactivated 99.9% of *S. aureus* within 2 min and formed almost the highest yield of organic chloramines simultaneously which was quite stable during the experiment. Contrarily, it took more than 4 h for free chlorine to kill 99.9% of *M. mucogenicum,* although organic chloramines formation reaction still finished with 2 min. It implied that organic chloramines were not the by-products of fatal reaction of *M. mucogenicum*. So it could be hypothesized that chlorine disinfectants reacted with extracellular substances of mycobacteria rapidly and formed organic chloramines, which did not affect the metabolism directly.

Lipid Analysis

M. mucogenicum cell configuration was characterized by a much higher ratio of statured fatty acids in lipid profile which was quite different from that of *S. aureus* (Table 1). *S. aureus* cell contained high percentage of unsaturated fatty acid (74.8%). In contrast, *M. mucogenicum* cell was dominantly constructed by saturated fatty acid (95.7%). The percentage of saturated fatty acids in *M. mucogenicum* was 4.26 times of that in *S. aureus*.

Table1. Comparison of Lipids Contents in *S. aureus* and *M. mucogenicum*

Lipid	Percentage of Total Lipid (%)	
	S. aureus	*M. mucogenicum*
10:0	1.61	0
12:0	3.88	0
14:0	4.81	0
15:0*	0	43.93
16:0	14.42	5.37
16:1w7c/16:1w6c	15.48	0
17:0 anteiso	0	9.82
17:1 w7c	39.03	0
18:0	0	1.6
18:1 w9c	16.09	0
18:2 w6,9c	0	1.22
19:0	0	32.74
19:1 w6c	4.2	0
20:0	0	1.23
In Summary		
Saturated Fatty Acid	19.23	95.7
Unsaturated Fatty Acid	74.8	1.22

Note. * represents sum of 15:0 anteiso and 15:0 iso.

According to Stewart's description[27], cell wall fatty acids had the carbon chain lengths of 15 or less and cell membrane included the fatty acids with carbon chain lengths greater than or equal to 16. Accordingly, both *M. mucogenicum* and *S. aureus* had saturated wall fatty acid configuration whereas all fatty acids in *S. aureus* cell membrane were unsaturated while *M. mucogenicum* cells only contained one kind of unsaturated fatty acids (18:2 w6,9c) and very limited percentage (1.22%) in the membrane.

DISCUSSIONS

The *M. mucogenicum* strain isolated from a real distribution system was confirmed to be highly chlorine-resistant. As far as we know, this is the first report of chlorine resistance of mycobacteria in the field of water supply in China. The species of *M. avium*[11,16], *M. gordonae*[8], *M. xenopi*[31], and others, which used to be frequently reported in other countries, were not detected in our study. The strains of mycobacteria detected in this location were all identified as *M. mucogenicum*. Falkinham believed that the system was one of the factors influencing mycobacteria recovery[28]. Thomson compared several methods for isolating *M. avium* and pointed out that the results would be affected with processing methods. Overall, the comprehensive

understanding of the prevalence and species diversity of mycobacteria in China's water distribution system still needs more research.

By comparison of the reported Ct value for *M. avium*, *M aurum*, *M. gordonae*, *M. chelonae*, and *M. fortuitum* determined by Taylor and Le Dantec[26,28], from 10 to 200 min·(mg/L) for 99.9% of cell death, *M. mucogenicum* had a similar chlorine resistance as *M. aurum*, *M. gordonae* and less resistance than *M. avium*, *M. chelonae* and *M. fortuitum.*

The findings from this study provided insight of chlorine disinfection mechanism. This study also evaluated the efficiency of various disinfection processes. According to the inactivation experiment of mycobacteria, chlorine dioxide performed significantly higher efficiency than free chlorine, while these two kinds of disinfectants showed almost the same effect on inactivation of *S. aureus.* It is well known that the addition reactions and substitution reactions to unsaturated bonds are easy to occur with free chlorine instead of chlorine dioxide. The researchers[32] also observed previously that free chlorine reacted with unsaturated fatty acids, destructed the cell configuration and killed them eventually. It could be therefore speculated that free chlorine inactivation mainly relied on attacking the unsaturated bonds and chlorine dioxide inactivation was mainly led by oxidation. Hence, chlorine dioxide was more effective than free chlorine when disinfecting the chlorine resistant bacteria like mycobacteria which had long-chain saturated fatty acid in membrane and cell wall. Nevertheless, the mechanism of the high inactivation efficiency of chlorine dioxide on mycobacteria needs further study.

Although the consumption of disinfectant and formation of organic chloramines showed negative effect to inactivation efficiency in the inactivation experiment, the analysis of chlorine consumption on two bacteria with different chlorine resistance indicated that higher amount of chlorine consumption was not the direct cause of the chlorine resistance of mycobacteria. The lipid analysis indicated that the richness of long-chain saturated fatty acid or rareness of unsaturated fatty acid in cell membrane might partly explain the higher chlorine resistance of *Mycobacteria* over *S. aureus*.

Previous research[10] on mycobacteria had demonstrated that growth in low-nutrient conditions resulted in higher chlorine resistance of mycobacteria. Taylor et al.[28] showed that water-grown *M. avium* cells were 10-fold resistant than that of the medium-grown cells. Massa's study on sensitivity of *E. coli* proved that the growth conditions, especially nutrient availability could alter the lipid composition of cells[33]. Thus, it was reasonable to conclude that the special cell skeleton of mycobacteria contributed to its chlorine resistance. Stewart[27] believed that lipid might act as a physical barrier reducing the rate of penetration and subsequent oxidization of internal cellular materials. *M. mucogenicum* contained long-chain saturated fatty acids. The structural properties led to more hydrophobic characteristic which prevented the ionic chlorine disinfectants from diffusing and permeating into the cell. Meanwhile, higher proportions of saturated fatty acid decreased the reactivity and membrane fluidity which might also limit the movement of chlorine disinfectants into the cell.

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