INTRODUCTION

Circulating water pipes in facilities such as baths and swimming pools are often contaminated with microorganisms. These microorganisms form biofilms that remain on the pipes and some biofilms are potential sources of infection. For example, Legionellosis is a severe respiratory disease caused by mostly by Legionella pneumophila, and some of the outbreaks have been correlated with the presence of biofilms (Abdel-Nour et al., 2013). Sodium percarbonate (SP) can be used as a disinfectant to wash these pipes (Patel et al., 2016). SP is an addition compound in which sodium carbonate and hydrogen peroxide are mixed in a ratio of 2:3 Mol; its aqueous 1 wt% solution is alkaline (pH 10.5). SP, in an aqueous solution, yields sodium carbonate and hydrogen peroxide, which degrade into water and oxygen; it presents a lower environmental burden than chlorine-based bleaching agents. The compound, in its solid state, also provides higher safety and operability compared to liquid hydrogen peroxide. Thus, SP is often used as a cleaner for sanitizing circulating water pipes and as bleaching reagents in laundry, although the bactericidal effect of SP is lower than that of chlorine-based agents. 2-[Bis(carboxymethyl)amino] propanoic acid-chelated copper (MGDA-Cu) was added to increase the effect of SP. The addition of 12 µM MGDA-Cu increased the bactericidal effect of 0.5 wt% SP against Staphylococcus aureus even in the presence of 0.3 wt% BSA, which is an experimental model of organic stain to protect bacteria from SP. MGDA-Cu was effective against Escherichia coli only in the absence of BSA and showed little effect against Bacillus subtilis. It enhanced the effect of SP to decrease the viscosity of sodium alginate, which is one of the major components of biofilms. The effect of MGDA-Cu on sanitization was also evaluated by 16S rRNA amplicon sequencing of the bacterial flora of the biofilm on an experimental model of a circulating water pipe. The structure of the bacterial flora was more influenced by a cleanser containing both MGDA-Cu and SP than a cleanser with only SP, suggesting that MGDA-Cu increases the sanitization effect.

Key words: MGDA-Cu / Sodium Percarbonate / Biofilm / Chelate.
hydroxyl and hydroperoxyl radicals. ROS are highly reactive and unselectively oxidizes the surrounding chemicals, such as proteins and DNA of microorganisms, which induces biological damages (Pecci et al., 1997; Jomova and Valko, 2011). Production of ROS by copper and hydrogen peroxide can be partly controlled by chelating agents. The chelating of copper by 2,2',2'',2'''-(ethane-1,2,1,2-diyldinitrilo) tetraacetic acid (EDTA) decreases the production of hydroxyl radicals but increases hydroperoxyl radicals in the presence of hydrogen peroxide (Hodes et al., 2018). 2-[Bis(carboxymethyl)amino]propanoic acid (MGDA) is a chelating agent with higher biodegradability than that of EDTA (Tandy et al., 2004; Cao et al., 2008). It degrades in the natural environment twice as fast as EDTA (Kos et al., 2003; Cao et al., 2007). It can form water-soluble complexes with polyvalent ions over a wide pH range from 2 to 13.5 (Pinto et al., 2014). In addition, it usually forms complexes with metal ions at a molar ratio of 1:1 (Pinto et al., 2014).

Microorganisms produce biofilms consisting of polysaccharides and proteins to adhere to surfaces and resist physical stresses. Alginate is an important matrix molecule of biofilm produced by Pseudomonas aeruginosa (Mann and Wozniak, 2012) and Azotobacter vinelandii (Clementi, 1997). It is degraded by radicals produced from hydrogen peroxide, which produces a lower molecular weight of alginate (Mao et al., 2012) and carboxylic acid (Li et al., 2010). SP has a bactericidal effect on Staphylococcus aureus and Pseudomonas aeruginosa biofilms equivalent to that of sodium hypochlorite disinfectant products (Lineback et al., 2018).

Here, the effect of MGDA-Cu on SP sanitization was evaluated by determining the survival of Staphylococcus aureus, Escherichia coli, and Bacillus subtilis. Furthermore, the effects of MGDA-Cu on SP in enhancing alginate degradation and in altering of the structure of bacterial flora of the biofilm adhering on an experimental model of a circulating water pipe were elucidated. The obtained results suggest that the addition of MGDA-Cu increases the sanitization effect of SP.

**MATERIALS AND METHODS**

**Preparation of MGDA-Cu**

MGDA-Cu was prepared from a mixture of 108 mM MGDA (Trion M liquid, BASF Japan Ltd., Tokyo, Japan) and 13 mM copper (II) sulfate pentahydrate (UPINORG, JX Metals Trading Co., Ltd., Tokyo, Japan). This mixture was stocked and used as a 13 mM MGDA-Cu solution for subsequent experiments.

**Assay of bactericidal effects**

The bactericidal effect of SP (SPC, Zhejiang Jinke Household Chemical Materials Co., Ltd., Zhejiang, China) with MGDA-Cu was evaluated by assessing its influence on S. aureus NBRC12732, E. coli NBRC 3972, and B. subtilis NBRC3134, which were purchased from NITE Biological Resource Center (Tokyo, Japan). Bacteria were grown in a soybean-casein digest agar with lecithin and polysorbate 80 (SCDLP) medium (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) overnight at 35°C. The bacterial solution with turbidity adjusted for the number of bacteria ranged from 10⁷ to 10⁸ CFU (colony-forming units)/mL (0.1 mL) was mixed with physiological saline solution (0.85 wt% NaCl) or 3 wt% bovine serum albumin (1 mL) and applied to 9 mL of 0.5 wt% SP in the presence or absence of 12 µM MGDA-Cu. The SP concentration (0.5 wt%) was determined according to the previous study showing that 0.12 wt% SP has little effect on the survival of S. aureus in the presence of 3 wt% bovine serum albumin (Hiura et al., 2010). The concentration of MGDA-Cu was determined based on the decomposition of hydrogen peroxide, suggesting ROS formation. A similar amount (5.9 mM) of 29 mM hydrogen peroxide was decomposed by 12 or 25 µM MGDA-Cu at 50°C for 30 min, whereas only 1.5 mM hydrogen peroxide was decomposed in its absence (data not shown). It is worth noting that copper is a highly toxic environmental pollutant (Yuan et al., 2014), and thus a lower concentration is preferable. The exposure time and temperature were altered because these three strains showed various tolerances against SP. After incubation with SP, the solution (1 mL) was neutralized by the addition of 9 mL sodium sulfite (1 wt% for SP). The surviving bacterial cells were diluted with phosphate-buffered saline and incubated on SCDLP agar plates for 48 h at 35 °C, and the CFU were counted.

**Viscosity degradation of sodium alginate**

The 1.5 wt% solution of sodium alginate (IL-6, KIMICA Corp., Tokyo, Japan) was incubated at 40 °C in the presence of 1 wt% SP and/or 25 µM MGDA-Cu. The viscosity was determined every 3 min. It was measured using a rotational viscometer (rotor No. 2 and 60 rpm rotor speed) defined by JIS 8803.

**Formation of biofilm on an experimental model of a circulating water pipe**

A biofilm was formed on the experimental model of a circulating water pipe under unsterilized conditions. The alteration of bacterial flora in the biofilm was analyzed to determine the enhancement of the sanitization effect of SP by the addition of MGDA-Cu. The model consisted of a lateral square-U-shaped plastic pipe with a length...
of 2 m and width of 10 cm (Fig. 1). The water pipe was covered with an aluminum foil to prevent algal growth, and microorganisms in the environment were grown to form a biofilm. Tap water containing 500 mg/L skimmed milk (70 L) was recycled using a pump with a flow speed and rate of 0.2 m/s and 0.4 L/s, respectively in the pipe at 16 °C for 10 days; the skimmed milk solution was replaced with a fresh solution after 7 days. The formed biofilm (wet weight 5 g) was used to analyze the bacterial flora by amplicon sequencing. The pipe with biofilm was washed with 70 L of tap water containing 0.5 wt% SP for 1 h; the skimmed milk solution was circulated in the same manner as described earlier to form a biofilm after the disposal of the cleaner. The formed biofilm was analyzed, and the pipe washed with 70 L of tap water containing 0.5 wt% SP and 12 µM MGDA-Cu for 1 h. After the disposal of the cleaner, the biofilm was allowed to form in the same manner, and the formed biofilm was analyzed.

**Amplicon sequencing of 16S rRNA gene**

Biofilms on the pipes of the experimental model of the circulating water pipe were collected 10 days after the start of the circulation cycle. DNA was extracted from the biofilms using ISOIL (Nippon Gene, Tokyo, Japan) according to the instructions of the manufacturer. The V4 region of the prokaryotic 16S rRNA was amplified using 515F/806R primers (Caporaso et al., 2011). Amplification products with six replicates per sample were sequenced using the MiSeq instrument and MiSeq reagent kit v2 (300 cycles) (Illumina, San Diego, CA, USA) by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan).

**RESULTS**

**Effect of MGDA-Cu on bacterial elimination by SP**

The effects of MGDA-Cu on the bactericidal effect of SP were evaluated in vitro using *S. aureus*, *E. coli*, and *B. subtilis*. Viable *S. aureus* cells decreased after SP treatment for 10 min at 35 °C (Table 1). The addition of MGDA-Cu increased the bactericidal effect, and no viable cells were found after 10 min of treatment. The addition of BSA, which is an experimental model of organic staining, weakened the bactericidal effect of SP. The number of cells that survived after SP treatment for 20 min in the presence of BSA (log10 5.0 CFU/mL) was almost equal to that after the 10 min treatment in the absence of BSA (log10 5.2 CFU/mL). The addition of MGDA-Cu also increased the bactericidal effect in the presence of BSA. No viable cells survived after 40 min of treatment, although log10 3.0 CFU/mL cells survived in the absence of MGDA-Cu. These results show that the addition of MGDA-Cu increases the bactericidal effect of SP against *S. aureus*.

*E. coli* was more labile in the presence of SP than *S. aureus*, and no viable cells were observed after the SP and MGDA-Cu treatment for 1 min in the absence of
BSA increased the resistance of *E. coli* to SP; however, all the cells were eliminated after 10 min of treatment, both with SP alone and SP and MGDA-Cu. *E. coli* was too labile against SP to elucidate the effect of MGDA-Cu under the experimental conditions. Most *B. subtilis* cells survived after treatment with SP in BSA for 30 min at 35 °C and MGDA-Cu showed little effect on the bactericidal activity of SP (data not shown). The temperature was increased to 60 °C to evaluate the effect of MGDA-Cu. However, the addition of MGDA-Cu showed only a faint effect on the bactericidal activity (Table 3).

Gram-positive bacteria, such as *B. subtilis* and *S. aureus*, are more resistant to ROS than gram-negative bacteria, including *E. coli* (Foster et al., 2011), since gram-positive bacteria possess thicker cell walls to protect themselves than gram-negative bacteria. The effect of MGDA-Cu on the bactericidal activity of SP may depend on the bacterial tolerance against ROS and their environments.

### Effect on the viscosity of sodium alginate

Alginate is a matrix molecule of biofilm that provides protection to microorganisms. Thus, degradation of alginate can increase the bactericidal effect of SP. The effect of MGDA-Cu on the depolymerization of alginate by SP was evaluated (Fig. 2). A considerable decrease in viscosity was observed in the presence of both SP and MGDA-Cu compared to SP alone. This result suggests that MGDA-Cu also increases the depolymerization effect of SP to enhance the bactericidal effect of SP.

### Effects on the structure of bacterial flora of the biofilm

To evaluate the effect of MGDA-Cu *in vivo*, prokaryotic 16S rRNA genes of the biofilm on the experimental model of circulating water pipes were analyzed by
FIG. 2. Effect of SP and MGDA-Cu on the viscosity of sodium alginate. Sodium alginate (1.5 wt%) was incubated in the presence of 1 wt% SP (closed triangle), 25 μM MGDA-Cu (closed square), or 1 wt% SP and 25 μM MGDA-Cu (open circle).

TABLE 4. Number of sequences assigned to bacterial phyla.

| Phylum              | None  | SP     | SP+MGDA-Cu |
|---------------------|-------|--------|------------|
| Proteobacteria      | 22038 | 20842  | 21709      |
| Bacteroidetes       | 12046 | 11329  | 19101      |
| Firmicutes          | 2179  | 818    | 1771       |
| Actinobacteria      | 212   | 383    | 888        |
| Verrucomicrobia     | 287   | 153    | 206        |
| Armatimonadetes     | 28    | 12     | 45         |
| Cyanobacteria       | 0     | 7      | 21         |
| Chloroflexi         | 44    | 32     | 0          |
| Total               | 36834 | 33576  | 43741      |

FIG. 3. Composition ratio of the bacterial flora in the biofilm. The biofilms on the experimental model of circulating water pipe before washing (none), after washing by SP (SP), and the mixture of SP and MGDA-Cu (SP+MGDA-Cu) were analyzed by amplicon sequencing of prokaryotic 16S rRNA genes. (A) The ratio of bacterial phyla shown in Table 4. (B) Proteobacteria phylum is further divided into four classes. (C) Bacteroidetes phylum is further divided into four classes and others which cannot be classified.
amplicon sequencing (Fig. 3). The sequencing reads 36834, 33576, and 43741 sequences of the obtained biofilms formed without washing, after SP and SP with MGDA-Cu washings, respectively (Table 4). These obtained sequences were assigned to eight bacterial phyla, and most sequences were assigned to phyla Proteobacteria and Bacteroidetes. Circulation of the mixture of SP and MGDA-Cu induced larger compositional changes compared to that of SP alone (Fig. 3A). Larger compositional changes were also observed in the classes of phyla of Proteobacteria (Fig. 3B) and Bacteroidetes (Fig. 3C) by the addition of MGDA-Cu. Bactericidal agents after microbial composition (Chatzi giannidou et al., 2020). The addition of MGDA-Cu to SP increases the bactericidal activity of SP and thus may induce a larger compositional change in the structure of bacterial flora of the biofilm.

**DISCUSSION**

Biofilms on circulating water pipes are a potential source of infection, and thus the washing methods should be further investigated for efficient disinfection. In this study, the effect of MGDA-Cu on the sanitization effect of SP was evaluated. The addition of MGDA-Cu increased the bactericidal effect of SP against *S. aureus* (Table 1) and *E. coli* (Table 2), however, MGDA-Cu showed little effect against *B. subtilis* (Table 3). The increase in the bactericidal effect of SP by MGDA-Cu seems to occur under limited conditions. In addition to the direct bactericidal effect, MGDA-Cu facilitated the depolymerization of alginate by SP (Fig. 2). The depolymerization of alginate that holds microorganisms together should degrade the biofilm to remove them. The addition of MGDA-Cu may increase the sanitization effect of SP by increasing both of the bactericidal effect and removal action. The improved sanitization effect of SP owing to the addition of MGDA-Cu was confirmed by analyzing the bacterial flora in biofilms on an experimental model of a circulating water pipe. MGDA-Cu induced larger compositional changes in the structure of the bacterial flora in the biofilm. The obtained results suggest that MGDA-Cu increases the sanitization effect of SP. Further investigation is required to assess the disinfectant efficiency of MGDA-Cu in actual facilities using circulating water pipes.

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