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Note

Effect of Sodium Hypochlorite on the Biofilms of *Aeromonas hydrophila*, *Streptococcus mutans*, and *Yersinia enterocolitica*

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In this study, the effect of sodium hypochlorite on biofilm removal was evaluated using three bacterial strains; *Aeromonas hydrophila*, *Streptococcus mutans*, and *Yersinia enterocolitica*. For maximum biofilm removal in 10 min, sodium hypochlorite is required at 1.65, 0.83, and 0.41 g/l for *A. hydrophila*, *S. mutans*, and *Y. enterocolitica*, respectively. Resistance to sodium hypochlorite was increased by the biofilms of all three tested strains, while the change in bactericidal activity according to sodium hypochlorite concentration was strain-specific. Therefore, we aimed to determine the effective concentration of sodium hypochlorite required for hygiene, considering that higher concentrations are needed to remove biofilms than to kill cells.

Keywords: ***Aeromonas hydrophila*, biofilm, sodium hypochlorite, *Streptococcus mutans*, *Yersinia enterocolitica***

Biofilms provide a physical barrier against bactericidal chemicals and a unique environmental condition to change cell physiology, promoting resistance to stresses [1–4]. In strategies of hygiene, it is essential to kill the cells and remove biofilms because the residual biofilms serve a new niche for bacteria [5]. Sodium hypochlorite and ethanol have been used commonly as disinfectants because they are affordable, easy to handle, and applicable to many bacteria [6, 7]. Additionally, they do not select resistant bacterial strains [8]. Around 70% ethanol is commonly used as an antibacterial agent because of its effectiveness [6, 9]; however, the removal of bacterial biofilms by ethanol was more effective at around 40% [10].

In this study, biofilms of *Aeromonas hydrophila*, *Streptococcus mutans* GS-5, and *Yersinia enterocolitica* (KCCM 41657) were removed by sodium hypochlorite. *A. hydrophila* is a gram-negative pathogen infecting humans and other mammals and causes gastroenteritis [11, 12] and forms biofilms [13]. *S. mutans* is an important dental pathogen causing human dental caries [14]. Dental plaque, which is the biofilm of many bacteria including *S. mutans*, plays an essential role in causing dental diseases [15]. *Y. enterocolitica* is a foodborne pathogen and mainly causes gastroenteritis in a healthy host. Most serious symptoms develop in young children under the age of one [16]. It is also a good biofilm producer, and its robust biofilm formation requires rotating flagella [17].

A. hydrophila and *S. mutans* GS-5 were obtained from the Research Institute of LG Household & Health Care Co., Ltd. (Daejeon, Korea). Luria-Bertani (LB) and Brain Heart Infusion (BHI) medium were purchased from Becton, Dickinson, and Company (USA). Tryptone

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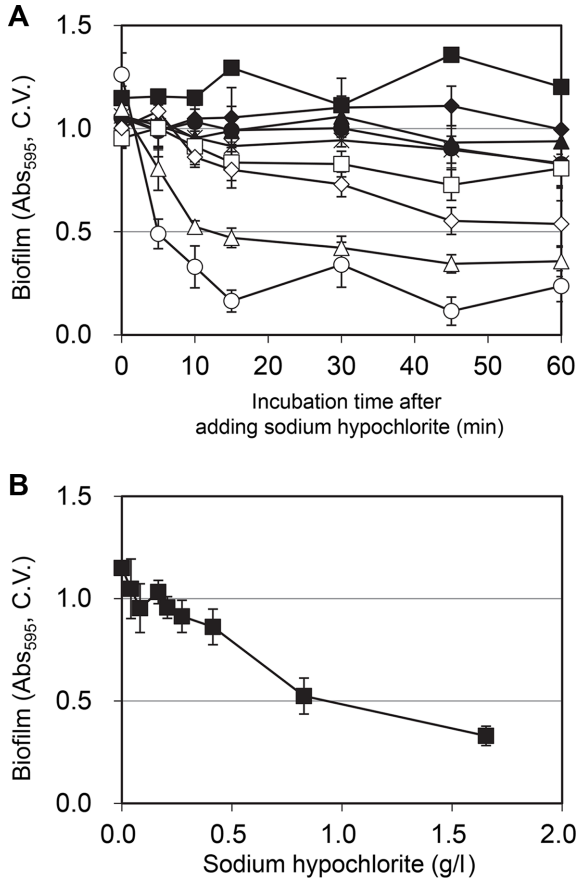

Fig. 1. Removal of biofilm mass of *A. hydrophila* after sodium hypochlorite treatment. (A) After biofilm production, sodium hypochlorite was added. The residual biofilm was analyzed quantitatively using crystal violet (C.V.). The tested concentrations of sodium hypochlorite used for the treatment were: control (■), 0.04 g/l (◆), 0.08 g/l (▲), 0.17 g/l (●), 0.21 g/l (×), 0.27 g/l (□), 0.41 g/l (◇), 0.83 g/l (△), and 1.65 g/l (○). (B) After 10 min of treatment (A), the result was selected, and the change of removal effect as per the concentration of sodium hypochlorite was shown.

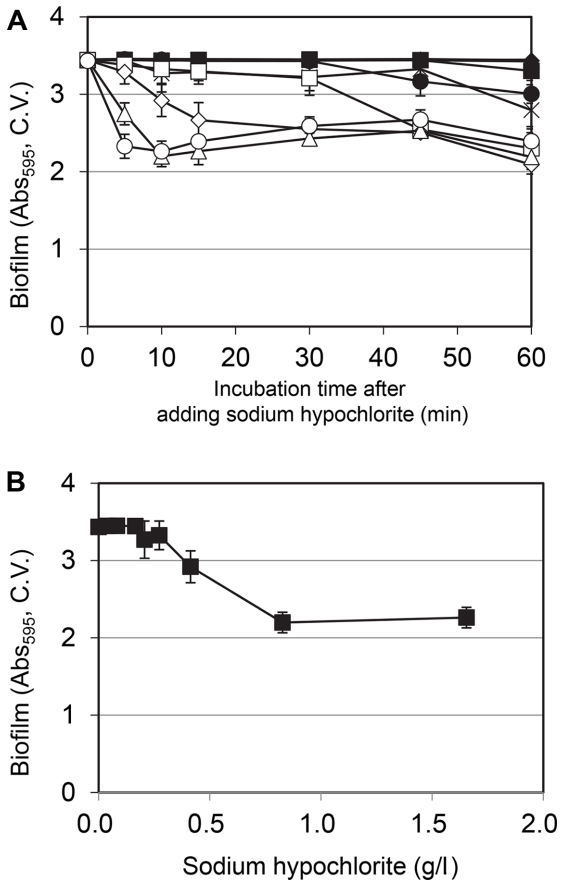

Fig. 2. Removal of biofilm mass of *S. mutans* after sodium hypochlorite treatment. (A) After biofilm production, sodium hypochlorite was added. The residual biofilm was analyzed quantitatively using crystal violet (C.V.). The tested concentrations of sodium hypochlorite used for the treatment were: control (■), 0.04 g/l (◆), 0.08 g/l (▲), 0.17 g/l (●), 0.21 g/l (×), 0.27 g/l (□), 0.41 g/l (◇), 0.83 g/l (△), and 1.65 g/l (○). (B) After 10 min of treatment (A), the result was selected, and the change of removal effect as per the concentration of sodium hypochlorite was shown.

Yeast Extract (TYE) and M63 Glucose Magnesium sulfate (M63GM) medium were prepared as described in a previous study [17]. Trypticase Soy Broth with Yeast extract (TSBY) medium was prepared as previously described by Kirov *et al.* [18]. Agar plates were prepared with 1.5% agar. The biofilms of *A. hydrophila* and *Y. enterocolitica* were produced according to Park *et al.* [10], and the biofilm of *S. mutans* was produced according to Ham and Kim [19]. Sodium hypochlorite (100 μl) was added to the microplates and incubated. Each well was rinsed three times, and the residual biofilms were analyzed quantitatively according to previous studies [17].

In order to remove around 50% and more biofilm in *A. hydrophila*, the concentration of sodium hypochlorite and the treatment time should be more than 10 min at 0.83 g/l (Fig. 1A). Treatment with sodium hypochlorite (1.65 g/l) for 15 min removed the maximum biofilm mass, leaving about 20% biofilm mass. The biofilm removal efficacy of sodium hypochlorite was proportional to its concentration (Fig. 1B).

For *S. mutans*, around 60% or more biofilm was remained after sodium hypochlorite treatment at 1.65 g/l for 60 min (Fig. 2A). The biofilm removal efficacy did not increase significantly by more treatment time after

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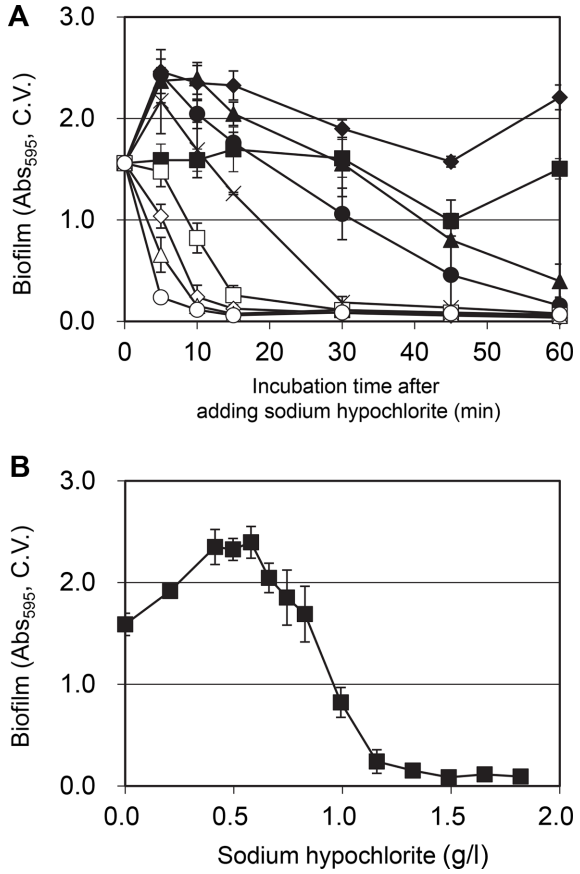

Fig. 3. Removal of biofilm mass of *Y. enterocolitica* after sodium hypochlorite treatment. (A) After biofilm production, sodium hypochlorite was added. The residual biofilm was analyzed quantitatively using crystal violet (C.V.). The tested concentrations of sodium hypochlorite used for the treatment were: control (■), 0.04 g/l (◆), 0.08 g/l (▲), 0.17 g/l (●), 0.21 g/l (×), 0.27 g/l (□), 0.41 g/l (◇), 0.83 g/l (△), and 1.65 g/l (○). (B) After 10 min of treatment (A), the result was selected, and the change of removal effect as per the concentration of sodium hypochlorite was shown.

10 min at 1.65 g/l or by more concentration than 0.27 g/l for 60 min. Since sodium hypochlorite did not remove biofilm well, the residual biofilm could be a severe hygiene problem for *S. mutans* (Fig. 2B).

Y. enterocolitica biofilms have a high sensitivity to sodium hypochlorite (Fig. 3A). The minimum concentration of sodium hypochlorite required to remove more than 90% of biofilm mass was 0.17 g/l for 60 min. Treatment with 1.16 g/l of sodium hypochlorite for 10 min killed 85% of the cells in the biofilm (Fig. 3B). Because *Y. enterocolitica* is a good biofilm producer [17], the removal of its biofilm is essential for hygiene.

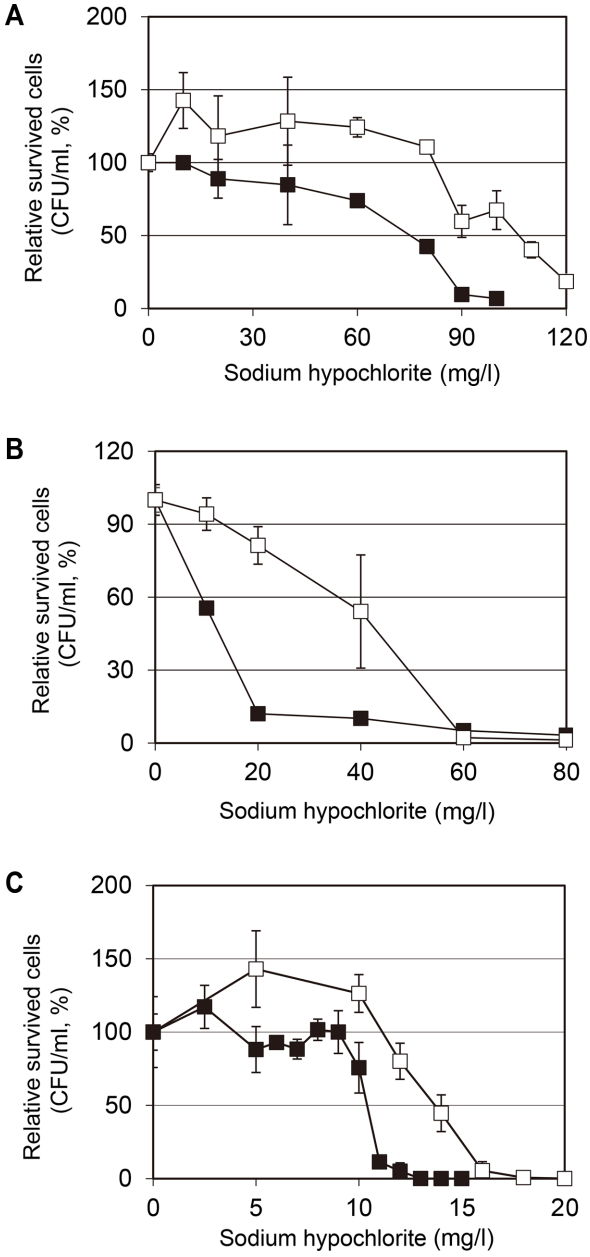

Fig. 4. Relative survival ratio of three *A. hydrophila* (A), *S. mutans* (B), and *Y. enterocolitica* (C) after sodium hypochlorite treatment. The planktonic growing cells (■) and the cells in the biofilms (□) were exposed to sodium hypochlorite for 10 min at room temperature.

The protective effect of the biofilm against sodium hypochlorite was analyzed quantitatively for the three strains (Fig. 4). For performing the survival test, the planktonic cells and cells in biofilm were incubated with sodium hypochlorite for 10 min at room temperature.

The number of viable cells was counted as colony forming units (CFU/ml) and the relative survived cells was calculated by comparing cell density from the cell culture without treatment of sodium hypochlorite. The data was the average of triplicate results.

In the planktonic cells of *A. hydrophila* (Fig. 4A), the number of survived cells gradually decreased up to 60 mg/l and the cells rapidly died at more than 60 mg/l. In the biofilm, cell death was not observed up to 80 mg/l, but the cells also rapidly died at more than 80 mg/l. These results suggested that biofilm increased the minimum sodium hypochlorite concentration required to kill cells. The planktonic cells of *A. hydrophila* were killed by 50–50,000 mg/l sodium hypochlorite within 1 min [20] or by 200 mg/l within 10 min [13]. However, the cells in the biofilms were killed by 2,000 mg/l sodium hypochlorite within 25 min [13]. Considering the difference in the results of the references [13, 20], the result of Fig. 4A shows the appropriate bactericidal activity of sodium hypochlorite against *A. hydrophila*, and clearly suggests a resistance efficacy of biofilm.

In the case of *S. mutans* (Fig. 4B), both the planktonic cells and the cells in the biofilm were killed proportionally as the concentration of sodium hypochlorite increases, but the killing efficacy increased rapidly in the planktonic cells. These results suggest that biofilms also increase the resistance of *S. mutans* against sodium hypochlorite.

When the *Y. enterocolitica* cells were treated with sodium hypochlorite (up to 10 mg/l) for 10 min, no killing effect was observed in planktonic and biofilm cells (Fig. 4C). The planktonic cells were more sensitive to sodium hypochlorite concentration change than the biofilm cells at the concentration more than 10 mg/l. To reduce cell number by around 90%, the concentration of sodium hypochlorite needed to be at least 11 mg/l for planktonic cells but at least 15 mg/l for cells in the biofilm, which was 36% higher than for planktonic cells. In a previous study [21], the bactericidal effect on *Y. enterocolitica* was observed at 10 mg/l sodium hypochlorite, which is similar to the result of this study.

Changes in survival ratio of *Y. enterocolitica* were different after treatment with sodium hypochlorite and ethanol. In the ethanol treatment, the biofilm increased the concentration at which the cells started to be killed [10]. In the sodium hypochlorite treatment, the concentration required for cell death was the same for both the planktonic cells and cells in the biofilm, but the bactericidal activity increased rapidly in the planktonic cells as the concentration of sodium hypochlorite increased. These different observations between sodium hypochlorite and ethanol suggested differences in the protective mechanisms of biofilm in *Y. enterocolitica*.

In the three strains tested in this study, the cells inside the biofilm showed higher resistance to sodium hypochlorite than the planktonic cells, and the change in bactericidal activity according to the sodium hypochlorite concentration showed various kinetics. The optimal hygienic conditions should be determined by the number of cells killed and biofilm mass removal efficiency. The concentration of sodium hypochlorite for biofilm mass removal was higher than the concentration for cell death. Therefore, the concentration of sodium hypochlorite should be sufficiently higher than the concentration required to kill bacterial cells in order to obtain an excellent hygienic effect against bacteria forming a biofilm.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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