Note

Inactivation of *Vibrio parahaemolyticus* Unattached and Attached to a Solid Surface in pH-Controlled Sodium Hypochlorite Solutions

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The inactivation of *Vibrio parahaemolyticus* cells that were unattached or attached to a polyethylene terephthalate (PET) disc in pH-controlled sodium hypochlorite (NaOCl) solutions was studied under turbulent conditions. No significant desorption of attached cells occurred at the free available chlorine (FAC) concentrations from 0.1 to 1.0 mg/l. The number of viable cells was estimated by microbial calorimetry. The logarithmic relative reduction of viable cells was proportional to the product of the FAC concentration and time. In the pH range of 5.6 to 9.3, the first-order inactivation rate constants for unattached and attached cells increased with decreasing solution pH. It was found that the rate constants for unattached cells were approximately 6 to 7 times higher than those for attached cells at all pH values examined. It was confirmed that attached cells were more resistant to NaOCl solutions than unattached cells even when accessibility of attached cells to HOCl/OCl⁻ was enhanced under turbulent conditions.

Key words : *Vibrio parahaemolyticus* / Inactivation of attached bacteria / pH-controlled sodium hypochlorite / Resistance to chlorine / Microbial calorimetry.

Vibrio parahaemolyticus has been known to be one of the major food-poisoning bacteria in Asia (Katsui et al., 1999; Urano et al, 2006; Wong et al., 2002). Since 1996, the increase in the incidence of *V. parahaemolyticus* infections has been reported worldwide, e.g., in North America, India, and Europe (Fournier and Ogata, 2005). The main cause of this food-poisoning is eating raw or undercooked seafood contaminated with the pathogen. Generally, *V. parahaemolyticus* cells are present on the surfaces of seafood and therefore can adhere to hard surfaces such as cutting boards and food-processing equipment, which in turn leads to cross-contamination. To prevent an outbreak of food poisoning, solid surfaces to which *V. parahaemolyticus* cells are attached should be disinfected effectively.

In the food industry, sodium hypochlorite (NaOCl) has been the most widely used disinfectant for more than 100 years, because it fulfills many requirements of the ideal disinfectant such as a broad antimicrobial

spectrum and rapid bactericidal action (Rutala and Weber, 1997). However, the efficacy of NaOCl is reduced markedly when bacteria are present on solid surfaces. LeChevallier et al. (1988) reported that biofilm bacteria grown on various solid surfaces are 150 to 3,000 times more resistant to NaOCl (at pH 7) than unattached (planktonic) cells under gentle agitation conditions. They suggested that transport of HOCl/OCl⁻ into the biofilm was a major rate-limiting factor. To elucidate the practical efficacy of NaOCl disinfection, it is necessary to investigate its antimicrobial activity against attached cells under stirring conditions where there is no diffusion limitation. In addition, it is desirable to assess the number of viable cells remaining on the solid surface without trying to detach them since complete recovery of attached cells is not expected (Camper et al., 1985; LeChevallier et al., 1988).

Microbial calorimetry is an established technique for monitoring the real-time growth of microorganisms in various environments such as liquid medium (Hashimoto and Takahashi, 1982), food (Koumoto et al., 1996), and solid medium (Koga et al., 2004). Calorimetry can

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measure changes in the heat evolved during microbial growth, producing a growth thermogram, $g(t)$. The $g(t)$ curve provides information about not only the growth activity but also the initial number of viable cells. The difference in the number of viable cells is reflected by the delay time in the $g(t)$ curve in the exponential growth phase. Based on this concept, the relative reduction of viable cells by NaOCl treatment can be estimated by the delay time of $g(t)$ curve.

The purposes of this study were twofold: 1) to determine whether *V. parahaemolyticus* cells attached to a solid surface in an NaOCl solution under turbulent conditions are more resistant than unattached cells and 2) to evaluate the number of surviving cells on a solid surface after NaOCl treatment without trying to detach them. Polyethylene terephthalate (PET), whose surface is smooth and inert to the NaOCl solution, was used as the model solid surface. Inactivation experiments were thus conducted on PET discs with tightly attached cells in pH-controlled NaOCl solutions under turbulent conditions.

V. parahaemolyticus NBRC 12711 was obtained from the National Institute of Technology and Evaluation (NITE, Chiba). *V. parahaemolyticus* was grown on tryptic soy broth (TSB) (Merck KGaA, Darmstadt, Germany) supplemented with 2% NaCl, and its pH was adjusted at 7.5 with 0.5M NaOH.

V. parahaemolyticus was cultured in 50ml of TSB with reciprocal shaking (120 oscillations per min) at 25°C for 24 h. The culture was transferred into a 50-ml centrifugation tube and the cells were harvested by centrifugation ($5,350 \times g$ for 10 min). After the supernatant was discarded, the collected cells were then washed twice with 20 ml of saline (0.9% NaCl). The washed cells were resuspended in 130 ml of saline to get a final OD₆₆₀ of 2.0.

A PET board (1 mm thickness) was purchased from SANPLATEC Co. Ltd. (Osaka). Discs (8 mm in diameter) were cut out from the PET board, and washed in ethanol with sonication for 15 min and then dried at 40°C. A NaOCl reagent containing min. 50,000 mg FAC/l was purchased from Kanto Chemical Co., Inc. (Tokyo). All other chemicals were of analytical grade and were purchased from commercial sources.

For preparation of *V. parahaemolyticus* cell-attached PET discs, a 5-ml aliquot of cell suspension and a piece of PET disc were put in a 25-ml glass vial, and it was sealed with a butyl rubber stopper. The vial was placed in a water bath at 25°C and shaken at 80 oscillations per min for 2 h. After being shaken for 2h, the PET disc was transferred into 16-ml test tube containing 2 ml of saline and washed by a vortex mixer to remove loosely attached cells. The PET disc was washed again in 2 ml of saline as mentioned above, and the disc was then

immersed in 1 ml of saline and used for inactivation experiments within 2 h.

The number of *V. parahaemolyticus* cells attached to the PET disc were enumerated with a scanning electron microscope (S-3400N, Hitachi High-technologies) as described previously (Takahashi and Fukuzaki, 2012).

In microbial calorimetry, $g(t)$ curves were measured at various initial numbers of viable *V. parahaemolyticus* cells (N'_0) inoculated in the suspension or on the PET disc. For unattached cells, serial decimal dilutions of the cell suspension were made, and an 80- μ l aliquot was inoculated in 5 ml of TSB in a 30-ml glass vial with a screw cap. For attached cells, the PET disc with the attached cells was placed was inoculated in a 30-ml glass vial containing 5 ml of TSB medium. The vial was incubated in a microbial calorimeter (Antares, the Keihanna Academy of Science and Culture, NPO) at 25°C (Takahashi and Fukuzaki, 2012). The $g(t)$ curve was obtained and analyzed by a supplemented computer program (BPCL24ch ver.20130926, The Keihanna Academy of Science and Culture, NPO, Kyoto). Here, a lag time in the $g(t)$ was prolonged with a decrease in the N'_0 . As a result, the incubation time (t_a) at which the $g(t)$ curve reached a definite value α (α is arbitrarily selected value) correlated with the N'_0 . In the present study, α was arbitrarily set to be 20 μ V. The standard curve depicting the relationship between the N'_0 of unattached cells and t_a was constructed.

Inactivation experiments were conducted in 0.1M phosphate-buffered saline (PBS) solutions at pH 5.6 to 9.4. For unattached cells, a 50- μ l aliquot of the cell suspension (OD₆₆₀ = 2.0) was diluted with 50 ml of the PBS solution. First, a 5-ml aliquot of the diluted cell suspension or a piece of the cell-attached PET disc was put into a 13-ml glass test tube (16.5 mm in diameter) containing 5 ml of the pH-controlled PBS. Secondly, a freshly prepared NaOCl solution of 100 mg FAC/l was added into the glass test tubes at final concentrations of 0.1 to 1.0 mg/l. Immediately after NaOCl was added, the test tube was vigorously mixed for 10 s, and then the same volume of 8 mM sodium thiosulfate as that of NaOCl solution was added into the test tube to terminate oxidation. The number of surviving cells in the suspension or on the PET disc after NaOCl disinfection was determined by the microbial calorimetry as described above.

The Chick-Watson law (Chick, 1908; Watson, 1908) was used to determine the rate of inactivation of *V. parahaemolyticus*.

$$\log (N/N_0) = -kCT \quad (1)$$

where N_0 is the initial number of viable cells, N is the number of surviving cells at time T , C is the FAC concentration, and k is the first-order inactivation rate constant of cells. It was generally observed that the

FIG. 1. Relationships between the logarithm of the initial number of viable cells inoculated (N_0) and the incubation time (t_a) giving the growth thermogram value of 20 μ V for unattached cells (A) and attached cells (B). Solid lines were obtained with linear regression analysis. Bars denote the standard deviation.

inactivation of microorganisms followed the first-order kinetics with regard to the product of C and T (CT value). Since the CT value was the unit of bactericidal activity, the logarithmic relative reduction of viable cells was plotted against the CT value.

After being in contact with PET disc for 2 h, *V. parahaemolyticus* cells were tightly attached to the PET disc. SEM observation showed that those cells were directly attached to the PET surface, without forming a multilayer biofilm (data not shown). In addition, no observable extracellular polymeric substances (EPS) were seen. From images of 9 points on the PET surface collected by SEM observation, the mean number of cells on the PET disc was calculated to be $5.4 \times 10^4 \pm 1.6 \times 10^4$ cells/mm², and the total number of cells attached to two sides of the PET disc was estimated to be 5.4×10^6 cells/disc.

Figure 1 shows the relationships between the logarithm of N_0 and t_a for unattached cells (A) and attached cells (B) obtained by microbial calorimetry. A good linear relationship was observed in each culture system. The results for unattached cells and attached cells gave the following relationships:

$$\log N_0 = 10.8 - 0.46t_a \quad (R^2 = 0.997) \quad (2)$$

(unattached cells)

FIG. 2. Inactivation of *V. parahaemolyticus* cells unattached (A) and attached (B) to PET discs by the pH-controlled NaOCl solutions as a function of CT values. Solid lines were obtained with linear regression analysis. Initial FAC concentrations of 0.1 to 1.0 mg/l were used. Symbols: $\circ$, pH 5.6; $\blacktriangle$, pH 7.4; $\diamond$, pH 8.3; $\blacksquare$, pH 9.4.

$$\log N_0 = 11.2 - 0.52t_a \quad (R^2 = 0.955) \quad (3)$$

(attached cells)

These relationships also showed that a 1-log unit reduction of N_0 for unattached cells and attached cells corresponded to the time of 2.2 h and 1.9 h in the t_a , respectively.

Figure 2 compares the logarithmic relative reduction of surviving cells ($\log N/N_0$) for unattached cells (A) and attached cells (B) as a function of CT value. Inactivation experiments were conducted at 25°C for 10 s under turbulent conditions. At all pH values examined, unattached and attached cells were inactivated according to a pseudo-first-order reaction (eq. 1). In each experiment, a lag phase was not observed. The rate of inactivation for unattached and attached cells increased with decreasing pH from 9.4 to 5.6. In the pH range of 5 to 10, FAC exists in two different forms in an aqueous solution, i.e., HOCl and OCl⁻. It is believed that HOCl is the more active species in the bactericidal action (Brazis et al., 1958; Fair et al., 1948; Fukuzaki et al., 2007). Against *Escherichia coli* in an aqueous solution, HOCl is 50 to 80 times more effective than OCl⁻ as a disinfectant (Morris, 1966; Scarpino, 1972). The proportion of HOCl at pH 9.4 and 5.6 increases from 1.2% at pH 9.4 to 98.8% at pH 5.6 (calculated by $pK_a = 7.5$). These facts indicate that HOCl is more effective against not only unattached cells but also attached cells.

TABLE 1. The inactivation rate constants estimated for unattached and attached cells

pH	k_{unatch}	k_{atch}	$k_{\text{unatch}} / k_{\text{atch}}$
	(l/mg·s)		
5.6	1.23	0.18	6.8
7.4	0.79	0.11	7.2
8.3	0.40	0.07	5.7
9.4	0.19	0.03	6.3

It was noted that unattached cells were inactivated more rapidly than that of attached cells. In Fig.2, at pH 5.6, approximately a 3-log unit reduction for unattached cells could be achieved at a *CT* value of 2.5 mg·s/l, while for attached cells, only a 0.4-log unit reduction was observed. These data indicated that the cells attached to the PET disc were more resistant to the action of HOCl than unattached cells.

The inactivation rate constants estimated for unattached cells (k_{unatch}) and attached cells (k_{atch}) are summarized in Table 1. In the pH range of 5.6 to 9.4, the k_{unatch} values were approximately 5.7 to 7.2 times higher than the k_{atch} values. Compared with unattached cells, attachment of cells to the PET disc reduces the surface area of cells that come in contact with NaOCl solution even when interfacial turbulence was enhanced. This is probably responsible for the deceleration of the actions of FAC molecules.

In the present study, it was confirmed that *V. parahaemolyticus* cells attached to a PET surface were less affected by NaOCl disinfection under turbulent conditions than unattached cells. Therefore, for achieving certain disinfection levels, it is necessary to load a higher *CT* value to cells on a solid surface than to cells in a solution. More comprehensive study will be necessary to elucidate the difference between the susceptibility of unattached and attached cells to NaOCl solution.

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