

## Article

# The Mechanism of Chlorine Damage Using Enhanced Green Fluorescent Protein-Expressing *Escherichia coli*

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**Abstract:** This study investigated how chlorine inactivates and damages *Escherichia coli* cells. *E. coli* that had transformed to express enhanced green fluorescent protein (EGFP) at the cytoplasm was treated with chlorine. Damage to the cell membrane and cell wall was analyzed by measuring the fluorescence intensity of the leaked EGFP, then accounting for the fluorescence deterioration. At pH 7, *E. coli* was lethally damaged after treatment with chlorine, but significant leakage of EGFP was not observed. In contrast, significant leakage of EGFP was observed at pH 9, even though *E. coli* was not as inactivated as it was at pH 7. Flow cytometry was used to confirm the fluorescence intensity of the remaining EGFP inside the cells. No significant fluorescence loss was observed in the cells at pH 7. However, at pH 9, the fluorescence intensity in the cells decreased, indicating leakage of EGFP. These results suggest that hypochlorous acid inactivates *E. coli* without damaging its cell membrane and cell wall, whereas the hypochlorite ion inactivates *E. coli* by damaging its cell membrane and cell wall. It was possible to confirm the chlorine damage mechanism on *E. coli* by measuring the fluorescence intensity of the leaked EGFP.

**Keywords:** *Escherichia coli*; damage mechanism; chlorine; EGFP; flow cytometry

## 1. Introduction

During water treatment, disinfectants are used to inactivate or sufficiently suppress the proliferation of pathogenic microorganisms. For example, chlorination, one of the most widely used disinfection methods, employs the powerful oxidizing capacity of chlorine to kill pathogenic microorganisms. This technique is relatively inexpensive; moreover, a small amount of chlorine can disinfect a large amount of water. Furthermore, the disinfection effect of chlorination persists even in the water distribution system. In solution, chlorine exists in several main forms, the relative contents of which depend on solution pH. Specifically, the redox potential of sodium hypochlorite, a substance widely used for chlorination, is a straight line with an inflection point at a specific pH corresponding to the  $pK_a$  of hypochlorous acid (7.537). In this case, hypochlorous acid is the main species at  $pH \leq 7.5$ , and the hypochlorite ion is the main species at  $pH \geq 7.5$  [1].

*Escherichia coli* has been used as a model bacterium to prove the disinfection effects of chlorine. However, it is not clear whether chlorine affects other pathogenic microorganisms in the same manner as *E. coli*. To clarify this, it is necessary to first understand where in the cell chlorine acts and how much damage it inflicts on *E. coli*. The mechanism of chlorine damage to pathogenic microorganisms may be determined by clarifying the mechanism of chlorine damage on *E. coli*. Various studies have been conducted with this aim. By measuring oxygen uptake, Albrich and Hurst [2] showed that chlorine decreases *E. coli* cell viability before the onset of appreciable respiratory loss. Dukan and Touati [3] investigated mechanisms of killing *E. coli* via un-ionized hypochlorous acid (HOCl), by identifying

which genes are involved in disinfection resistance. The effects of chlorination have also been evaluated by measuring enzyme activity. For example, Tryland et al. [4] investigated the effect of chlorine on the  $\beta$ -D-galactosidase activity of *E. coli* using the  $\beta$ -D-galactosidase assay.

While elucidating the mechanisms of damage by disinfection on microorganisms, it is important to evaluate the damage to specific sites of the microorganisms. In some cases, pathogenicity of microorganisms is associated with specific sites on the microorganisms. Characterization of the damage on specific sites leads not only to understanding of damage mechanisms, but also greater certainty in the efficacy of disinfection, and two methods have been used to characterize the damage to specific sites. The first method evaluates leakage of cell components. For example, chlorine changes the *E. coli* cell membrane in a manner that leads to the leakage of proteins and RNA from cells [5]. Diao et al. [6] showed that when the cellular material released after disinfection accumulates on the filter, it is hardly noticeable in the chlorinated sample. The second method evaluates the permeability of certain substances after disinfection treatment. Using 2-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), Sips and Hamers [7] evaluated the cell wall permeability of *E. coli* after treatments with myeloperoxidase, hydrogen peroxide, and chloride ions. Their results show that the bacteria are killed through an increase in the permeability of the bacterial cell wall. Certain procedures, such as LIVE/DEAD and SYBR Green I (SGI)/propidium iodide (PI) staining evaluate membrane damage by staining the nucleic acids [8–10]. Ramseier et al. [10] investigated membrane damage in bacteria in drinking water using SGI/PI and flow cytometry, and reported that chlorine is responsible for such membrane damage. Some mechanisms of damage are characterized by measuring the concentration of bacteria grown simultaneously on non-selective and selective agars, then considering the function of inhibiting substances in the selective agars [11–14]. Using this agar method combined with propidium monoazide (PMA) treatment that detects DNA only from live cells, Kazama and Otaki [14] showed that chlorination causes outer membrane damage. Another approach is to combine the assessments of leakage of internal components and permeability to external substances. For example, Tsuchido and Katsui [15] investigated how heat damages *E. coli* by measuring the leakage of lipopolysaccharide (LPS) and alkaline phosphatase (ALP) from the bacteria and its permeability to crystal violet. They showed that heat treatment induces the release of cell constituents and the penetration of crystal violet through part of the outer membrane. Virto et al. [16] investigated how chlorine induces *E. coli* damage by measuring the leakage of intracellular substances and staining with PI. It is noteworthy that PI is relatively small (MW: 668.4 Da) compared to RNA, proteins, and nucleic acids. However, the results of leakage and permeability had the same profile. Cho et al. [17] evaluated the damage of several disinfectants on *E. coli* by measuring the protein leakage and penetration of ONPG. They reported that free chlorine induces much less protein leakage than ozone, and that it results in very little surface damage to *E. coli*. Aside from proteins and nucleic acids, the leakage of ATP has also been studied, and a relationship between membrane damage (using SGI/PI) and leakage of ATP has been shown [18,19].

In evaluating the leakage of cell components, it is necessary to clearly prove that leakage comes from a specific substance inside cells and to show which part of the cell the substance is leaking from. Furthermore, it is necessary to examine the influence of external factors after leakage. In evaluating the permeability of cells to certain substances after disinfection treatment, the methods used have typically evaluated membrane damage; however, it has been difficult to confirm which part of the membrane is damaged.

In this research, we constructed *E. coli* expressing enhanced green fluorescent protein (EGFP). Microorganisms do not have the capability of expressing EGFP unless they are transformed. Therefore, EGFP is an excellent indicator of leakage from an internally produced substance. In addition, it is possible to express EGFP site-specifically in *E. coli* via recombination. Thus, it is an excellent means of localization [20,21]. Although green fluorescent protein (GFP) is stable [22], fading may occur, owing to various factors such as buffer, pH, and disinfectant [23–26]. In this study, we used EGFP to clarify the mechanism of damage by chlorine on *E. coli*. First, we confirmed the influence of the experimental conditions on the leaked fluorescence intensity of EGFP. Next, we determined the degree of damage by

chlorine to the cell membrane and cell wall of *E. coli* by expressing EGFP in the cytoplasm, treating the bacterium with chlorine, and measuring the fluorescence intensity of EGFP released from the cell. We validated the results of this experiment by confirming the fluorescence intensity inside the cell using a flow cytometer. The speciation of chlorine forms (hypochlorous acid and hypochlorite ion) was controlled by varying the solution pH, and the effect of this speciation on *E. coli* cells was evaluated.

## 2. Materials and Methods

### 2.1. Competent Cells

In this study, *E. coli* DH5 $\alpha$  (Takara Bio Inc., Japan), a strain with high transformation efficiency, was used. A single colony was incubated in Luria-Bertani (LB) medium (LB Broth, Difco Co., USA) at 37 °C overnight with shaking. An aliquot (1 mL) of the culture suspension was added to the LB medium (40 mL) and incubated at 37 °C for 2.5 h with shaking. After 5 min of cooling on ice, the culture was centrifuged at 1472 g for 5 min. The supernatant was discarded and 20 mL of ice-cold transformation buffer (TFB) (10 mmol L<sup>-1</sup> MES-KOH (pH 6.2), 100 mmol L<sup>-1</sup> KCl, 45 mmol L<sup>-1</sup> MnCl<sub>2</sub>, 50 mmol L<sup>-1</sup> CaCl<sub>2</sub>, 15% glycerol) was added. After 30 min of cooling on ice, the suspension was centrifuged at 1472 g for 5 min. The supernatant was discarded and 4 mL ice-cold TFB was added. An aliquot (100  $\mu$ L) was transferred into a sterilized tube and, after freezing at -20 °C for 2 h, the cells were stored at -80 °C.

### 2.2. Transformation

*E. coli* DH5 $\alpha$  was transformed with pEGFP (Takara Bio Inc., Japan) to express EGFP (MW: ca. 30,000) at the cytoplasm. A stock solution of pEGFP (10  $\mu$ L) was added to competent cells (100  $\mu$ L) of *E. coli*. After 6 min of cooling on ice, the mixture was placed in a 42 °C water bath for 3 min, then LB medium (1 mL) was added. An aliquot was applied onto LB agar medium (LB Broth and Bacto Agar, Difco., USA) containing ampicillin (Wako Co., Japan) (final concentration: 10 ng  $\mu$ L<sup>-1</sup>) and incubated at 37 °C for 20 h. As a control, pure water was added instead of pEGFP and treated as described above. *E. coli* of the control sample failed to grow on LB agar medium containing ampicillin. On the other hand, bacteria transformed with pEGFP (further referred to as transformed *E. coli*) formed multiple colonies, which was indicative of the selective culturing of ampicillin-resistant *E. coli*. An aliquot of the control sample was applied onto LB agar medium without ampicillin and incubated to afford untransformed *E. coli*.

### 2.3. *E. coli* Suspension

A colony of transformed *E. coli* was inoculated into LB medium (15 mL) containing ampicillin and incubated at 37 °C for 16–17 h with shaking. After centrifugation at 11,302 g for 5 min, the supernatant was discarded and the same amount of sterilized phosphate buffered saline (PBS) (pH 7.4, Nissui Pharmaceutical Co., Ltd.; Japan) was added and mixed. The untransformed *E. coli* suspension was made as described above using untransformed *E. coli* and LB medium without ampicillin.

### 2.4. Sensitivity of EGFP to Chlorine and pH

As the damage suffered by *E. coli* because of chlorination was assessed by monitoring the fluorescence intensity of EGFP leaked outside the cells, it was necessary to determine the effect of experimental conditions on the fluorescence intensity of leaked EGFP after chlorination. For this purpose, we prepared EGFP lysate and chlorinated it to probe the accompanying changes in fluorescence intensity.

#### 2.4.1. Fluorescent Protein Lysate

The transformed *E. coli* suspension was centrifuged at 11,302 g for 5 min. The supernatant was discarded, replaced with sterilized PBS, and mixed. After freezing the *E. coli* suspension at -80 °C for

2 h, it was thawed at room temperature. The supernatant was collected by centrifugation at 11,302 g for 5 min and filtered through a 0.20- $\mu$ m sterilized membrane filter (DISMIC-25AS, Toyo Roshi Kaisha, Ltd.; Japan) to obtain a fluorescent protein lysate. The pH 9 lysate was prepared as described above using PBS adjusted to pH 9 with sodium hydroxide (Wako Co., Japan), and pH 7 lysate was prepared in a similar way.

#### 2.4.2. Initial Samples

To closely simulate experimental chlorination conditions, the initial sample was prepared by mixing fluorescent protein lysate with untransformed *E. coli* suspension so as not to affect fluorescence intensity. The untransformed *E. coli* suspension was diluted with sterilized PBS until the absorbance at 600 nm was approximately 0.3. A fluorescent protein lysate (see Section 2.4.1) and the suspension were mixed for 5 min. The pH 9 initial sample was prepared as described above using pH 9 PBS, and the pH 7 initial sample was prepared in a similar way.

#### 2.4.3. Chlorine Disinfection

Initial samples (see Section 2.4.2) were collected for treatment with sodium hypochlorite. The sodium hypochlorite solution (5.0%, Wako Co., Japan) was diluted in pure water to make a stock solution (approximately 0.03%). A volume of the stock solution was added to the samples while mixing to achieve a final free chlorine concentration of between 0.15 and 0.63 mg L<sup>-1</sup>. The concentration of free chlorine was analyzed by the dialkyl-p-phenylenediamine (DPD) colorimetric method [27] after the chlorine stock solution was added. It was measured immediately after addition and immediately after the treatment, and the average value was the final free chlorine concentration. In the control, pure water was added instead of the chlorine stock solution. After a predetermined contact time (0–20 min), 5-mL sample aliquots were dechlorinated through the addition of a solution of sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O; Kishida Chemical Co., Ltd.; Japan) in pure water (50  $\mu$ L, 3 wt %).

#### 2.4.4. Fluorescence Intensity of EGFP

Aliquots (500  $\mu$ L) were collected before and after chlorine treatment and placed in 0.22- $\mu$ m filtration tubes (Ultrafree-MC centrifugal filter unit, Merck Millipore Ltd., USA). The samples were centrifuged at 1472 g for 5 min and the filtered samples were collected. After mixing, the samples were divided into four aliquots (100  $\mu$ L) and transferred into a 96-well plate (Thermo Scientific Nunc Microwell polymer bottom 96-well optical bottom plate, Thermo Fisher Scientific Co., USA) and the fluorescence intensity (482/516 nm) was measured using a BioTek Cytation 3 Cell Imaging Multi-Mode Reader (Bio Tek Inc., USA). Fluorescence intensity retention efficiency was calculated using Equation (1):

$$\text{Fluorescence intensity retention efficiency (\%)} = 100 \times I/I_0 \quad (1)$$

where  $I_0$  is the value obtained by subtracting the empty cell value (288) from the fluorescence intensity before chlorination, and  $I$  is the value obtained by subtracting the empty cell value from the fluorescence intensity after chlorination.

### 2.5. *E. coli* Damage Due to Chlorine Disinfection

#### 2.5.1. Initial Samples of *E. coli* Suspension

The transformed *E. coli* suspension was diluted with sterilized PBS until the absorbance at 600 nm was approximately 0.3. The pH 9 initial sample was made using pH 9 PBS, and the pH 7 sample was prepared similarly.

### 2.5.2. EGFP Expression Level

Initially, we determined how much EGFP was expressed in transformed *E. coli*. A 600- $\mu$ L aliquot of the initial sample (see Section 2.5.1) was collected and lysed using lysozyme (5.0 mg mL<sup>-1</sup>; Wako Co., Japan) in TBS (100 mmol L<sup>-1</sup> Tris, 1.5 mol L<sup>-1</sup> NaCl, HCl, Reverse Osmosis (RO) water). pH 9 TBS was prepared using sodium hydroxide. The *E. coli* suspension was centrifuged at 3537–9201 g for 5 min and the supernatant was discarded. Then, a 300- $\mu$ L volume of lysozyme solution was added and the mixture was mixed. An equal volume of TBS was then added and the mixture was placed at –80 °C for 10 min. After thawing at room temperature, the lysate was transferred into a 0.22- $\mu$ m filtration tube and centrifuged at 1472 g for 5 min. The filtered sample was mixed and divided into four parts and the fluorescence intensity (482/516 nm) from each 100  $\mu$ L aliquot was measured.

### 2.5.3. Chlorine Disinfection

The initial sample (Section 2.5.1) was placed in a bottle and treated as described above up to the chlorine disinfection step (Section 2.4.3).

### 2.5.4. Survival Ratio of *E. coli*

For *E. coli* cell counting, samples collected before and after chlorination were diluted with sterilized PBS and plated on the nonselective LB agar medium to quantify viable *E. coli* cells. The plates were incubated at 37 °C for 20–24 h, and the number of colonies that grew on LB agar after chlorination ( $N$ ) divided by the number of colonies that grew on LB agar before chlorination ( $N_0$ ) (i.e.,  $N/N_0$ ) was defined as the survival ratio. Log inactivation was defined using Equation (2):

$$\text{Log inactivation} = -\log (N/N_0) \quad (2)$$

### 2.5.5. Leakage of EGFP

Aliquots (500  $\mu$ L) were collected before and after chlorine treatment, and the fluorescence intensity of EGFP leaked out of the cell was measured as described above (Section 2.4.4). To compare the amount of leaked EGFP to the level expressed in cells, the EGFP leakage rate was calculated using Equation (3):

$$\text{Leakage rate of EGFP (\%)} = 100 \times (I - I_0)/I_L \quad (3)$$

where  $I$  is the value obtained by subtracting the empty cell value from the fluorescence intensity after chlorination,  $I_0$  is the value obtained by subtracting the empty cell value from the fluorescence intensity before chlorination, and  $I_L$  is the value obtained by subtracting the empty cell value from the fluorescence intensity after lysozyme treatment (Section 2.5.1).

Based on previous experimental results (Section 2.4), the fluorescence intensity retention efficiency could be determined from the CT (concentration of free chlorine  $\times$  contact time; mg min L<sup>-1</sup>) value. Therefore, maximum leakage was calculated by correcting the measured value using Equation (4):

$$\text{Maximum leakage} = 100 \times (\text{measured value} - 288)/(\text{fluorescence intensity retention efficiency}) \quad (4)$$

### 2.5.6. Remaining EGFP in Cells

Samples (1 mL) that were collected before and after treatment were analyzed by flow cytometry to measure the fluorescence intensity inside *E. coli* cells. Flow cytometer measurements were conducted in a CytoFLEX (Beckman Coulter, Inc. USA) equipped with a 50-mW laser emitting at a wavelength of 488 nm. Fluorescence intensity was collected at the Fluorescein isothiocyanate (FITC) channel (525/40-nm). Measurements were performed at a flow rate of 10  $\mu$ L min<sup>-1</sup>. All data were processed with the software CytExpert (Beckman Coulter, Inc. USA).

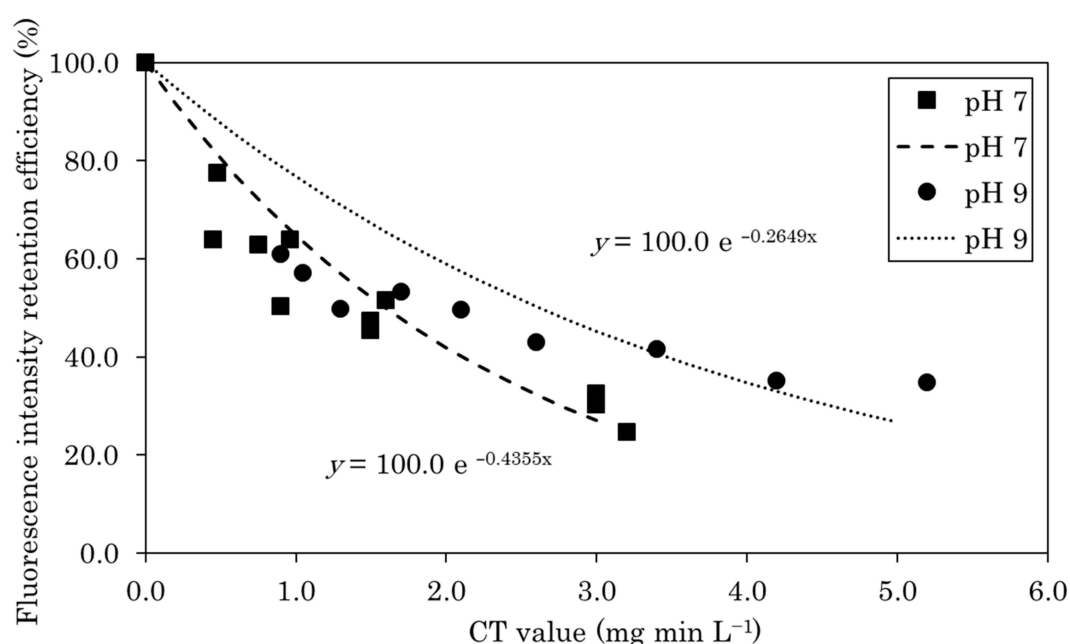
### 3. Results

#### 3.1. Sensitivity of EGFP to Chlorine and pH

The fluorescence intensities of EGFP were measured during chlorination at pH 7 and 9. Figure 1 shows the CT values and the corresponding fluorescence intensities of EGFP. The fluorescence intensity of EGFP, both at pH 7 and 9, decreased significantly after chlorination (Figure 1). The data were processed and fitted by a straight line assuming an intercept of zero as follows:

$$\text{Log}(I/I_0) = -k \times \text{CT value} \quad (5)$$

where  $I_0$  is the value obtained by subtracting the empty cell value from the fluorescence intensity before chlorination,  $I$  is the value obtained by subtracting the empty cell value from the fluorescence intensity after chlorination, and  $k$  is the first-order decay constant.



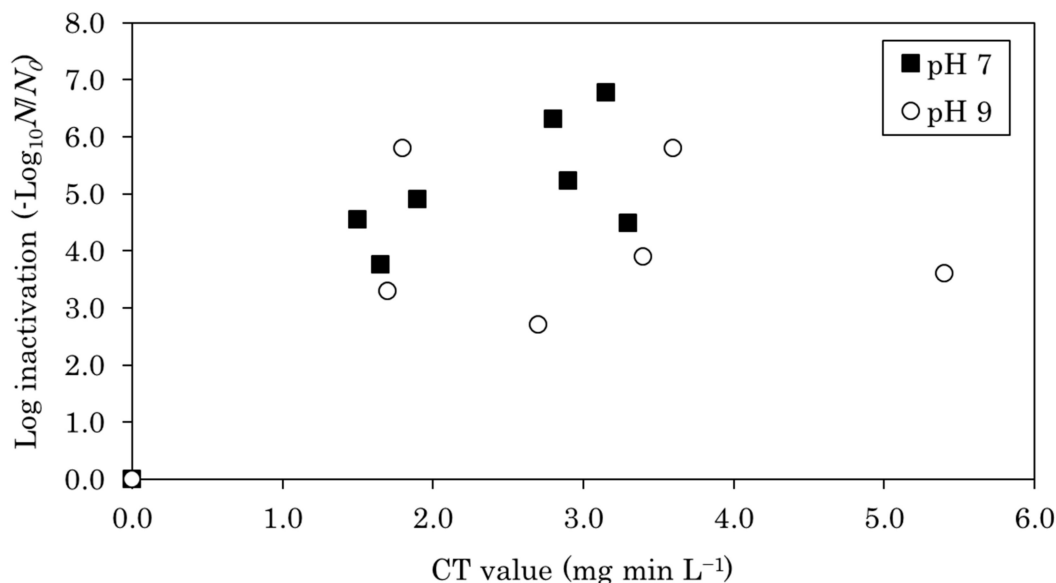
**Figure 1.** Fluorescence intensity retention efficiencies during chlorination at pH 7 and 9 plotted as functions of CT value. The fluorescence intensity reduction curve was calculated from the first-order decay constant.

The fluorescence intensity reduction curve, calculated from the first-order decay constant obtained by Equation (5) and shown in Figure 1, allowed the fluorescence intensity retention efficiency to be calculated based on the CT value during leaked EGFP quantitation in disinfection experiments. The calculated fluorescence intensity retention efficiency based on the CT value was applied to Equation (4), and calculated the maximum leakage in disinfection experiments.

#### 3.2. Log Inactivation

Figure 2 describes *E. coli* inactivation during chlorination, revealing that inactivation was more significant at pH 7 than at pH 9. At pH 7, it was inactivated by approximately 6 log when the CT value was 3, whereas at pH 9 it was inactivated by approximately 3 to 5 log. It has been shown that the hypochlorite ion has a lower disinfection effect than hypochlorous acid [28], which agrees with the results of this study.

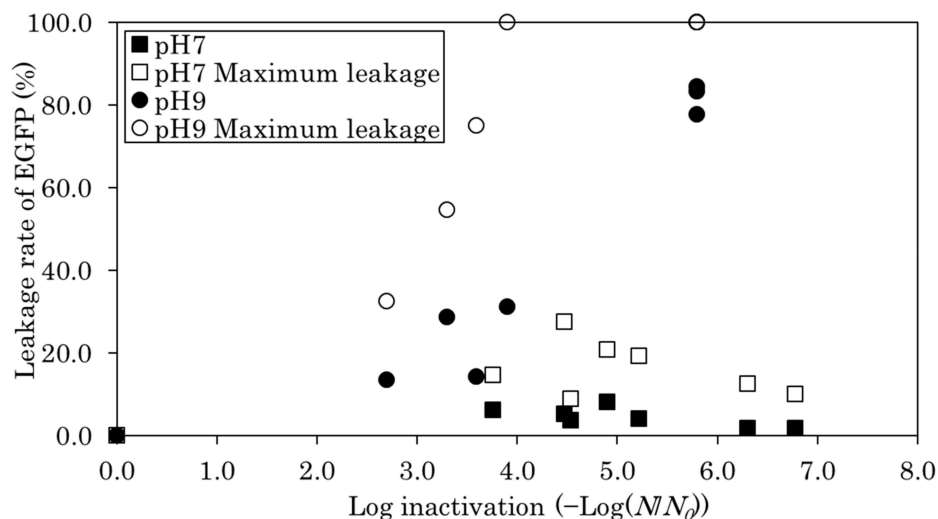




**Figure 2.** *E. coli* log inactivation during chlorination at pH 7 and 9, plotted as a function of the CT value.

### 3.3. Log Inactivation and Leakage Rate of EGFP

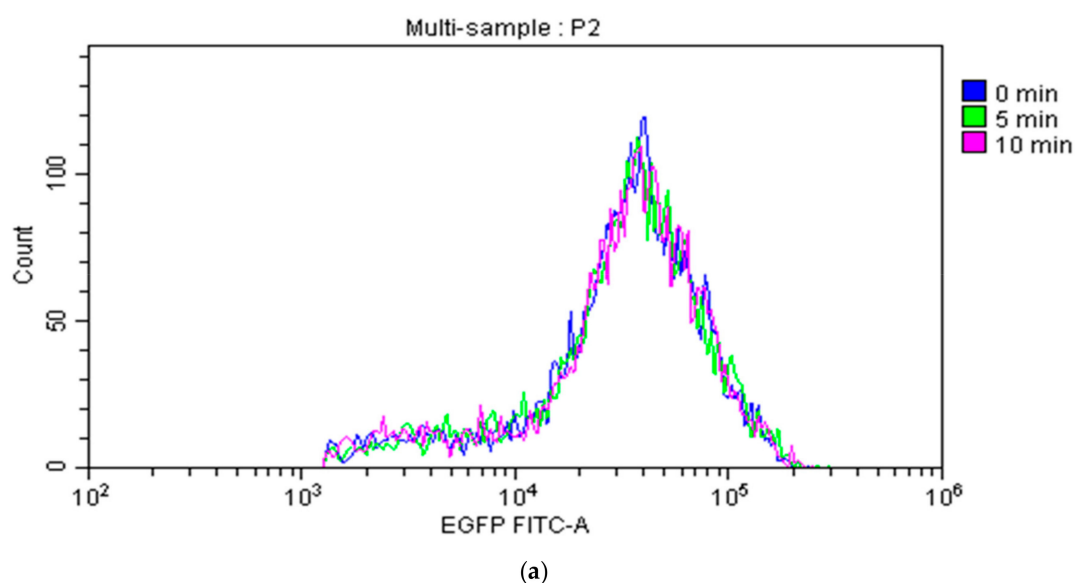
After lysozyme-induced cell lysis, the resulting fluorescence intensity of EGFP that leaked from the *E. coli* cytoplasm was assumed to be the total amount of EGFP inside the cell. The fluorescence intensity of leaked EGFP from *E. coli* cells after chlorination was compared with that of the total amount of EGFP. The leakage rate of EGFP is expressed in Figure 3. Figure 3 shows the change in the leakage rate of EGFP as the log inactivation increased. At pH 7, there was almost no leakage of EGFP from the cytoplasm regardless of the *E. coli* log inactivation (Figure 3). The measured leakage rate of EGFP remained at approximately 5%; however, considering the decrease of fluorescence intensity, the actual leakage rate may have been approximately 25%. This result suggests that chlorine did not significantly destroy the outside of *E. coli*, although it penetrated into the cell. At pH 9, the higher the *E. coli* log inactivation, the more EGFP leaked (Figure 3). The leakage rate of EGFP in 4 log inactivation was approximately 20%, based on the measured fluorescence value; however, considering the decrease of fluorescence intensity, the actual leakage rate may have been approximately 100%. The leakage rate of EGFP at 6 log inactivation was approximately 80% based on measured fluorescence value, and approximately 100% after correcting for the decrease of fluorescence intensity. These results suggest that chlorine damages the outside of *E. coli*. However, the leakage rate of EGFP before treatment was also higher at pH 9 than at pH 7. It is possible that the external surface of *E. coli* was destabilized when the pH was changed from pH 7 to 9. At pH 9, the hypochlorite ion reacted mainly with the outer surface of *E. coli*. The survival ratio was higher at pH 9 than that at pH 7, as described above. Therefore, we propose that, internally, *E. coli* was not as seriously damaged at pH 9 as it was at pH 7. It has been shown that the hypochlorite ion permeates the phospholipid bilayer of the cell membrane of gram-negative bacteria such as *E. coli* with difficulty [28]. This also makes it difficult to cause fatal damage. The results of this study also show that the hypochlorite ion is less lethal than hypochlorous acid. However, hypochlorite ions may act on the outer surface of the *E. coli* cell, which may also lead to fatal damage.



**Figure 3.** *E. coli* log inactivation and leakage rate of enhanced green fluorescent protein (EGFP) during chlorine treatment at pH 7 and 9. The leakage rate of EGFP that was calculated using measured values is plotted as a function of *E. coli* log inactivation. To consider the decrease in the fluorescence intensity of leaked EGFP, the maximum leakage rate was calculated from the CT value based on the results of Figure 1 and plotted.

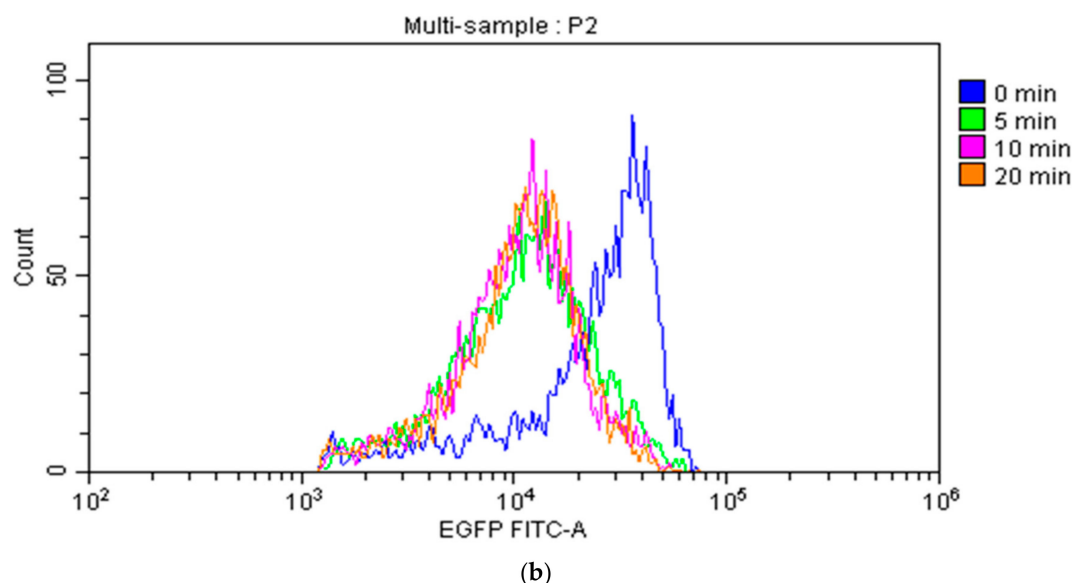
### 3.4. Fluorescence Intensity inside Cells

The internal fluorescence intensity was analyzed by flow cytometry. At pH 7, there was little change in the fluorescence intensity inside the cell during chlorination (Figure 4a). At pH 9, however, the change in internal fluorescence intensity due to chlorination was remarkable (Figure 4b). The maximum value of fluorescence intensity was lowered by chlorination, suggesting the leakage of EGFP from inside cells; this is supported by our results on the leakage rate of EGFP (Figure 3). However, fluorescence intensity did not change with chlorination time, a result that is also seen when the leakage rate of EGFP is plotted with the CT value on the horizontal axis (data not shown). Therefore, within the conditions of our experiments, much EGFP leaked at the timing of chlorine addition, with leakage proceeding slowly. This interpretation is consistent with the results of the leakage rate of EGFP (Figure 3).



**Figure 4.** Cont.





**Figure 4.** Changes in the fluorescence intensity of *E. coli* cells by chlorine at (a) pH 7 and (b) pH 9. Each histogram of 5000 gated events is shown as counts versus the log of fluorescence intensity. The color before chlorination is shown in blue and the colors after chlorination are shown in other colors for each treatment time.

#### 4. Discussion

In this study, we investigated whether the degree of damage to *E. coli* by chlorine can be measured by expressing EGFP in the cytoplasm then calculating the leakage in response to chlorine treatment. EGFP leakage was also confirmed by measuring the fluorescent intensity inside cells after chlorination. We investigated whether the degree of damage to *E. coli* depends on the form of chlorine and were able to confirm the leakage of EGFP in response to chlorine and measure the degree of damage to the cell membrane and cell wall of *E. coli*. Previous studies have also confirmed that fluorescence intensity is influenced by high concentrations of chlorine in solution, buffer, and various other external factors [23–26]. Here, we confirmed the effect of experimental conditions such as pH, chlorine concentration, and treatment time on the fluorescence intensity of EGFP. We confirmed that the degree of damage by chlorine depends on its pH-dependent form. When chlorine was predominantly present as hypochlorous acid, it gained entry to *E. coli* and inactivated cells with little destruction of the cell membrane and cell wall. As a certain amount of hypochlorite ions were present at pH 7, outer cell surface damage under these conditions was ascribed to these ions. In contrast, when the major form of chlorine was the hypochlorite ion, EGFP leaked, suggesting the destruction of the cell membrane and cell wall of *E. coli*. *E. coli* log inactivation was higher when the major form of chlorine was hypochlorous acid. Previous research suggests that hypochlorous acid can easily permeate the phospholipid bilayer comprising the cell membrane of *E. coli*, while the hypochlorite ion does not permeate as easily, making it difficult for the hypochlorite ion to fatally damage *E. coli* [28]. Our results show that hypochlorite ions do not cause fatal damage to *E. coli*, as does hypochlorous acid. However, these ions acted on the outer surface of *E. coli* to a greater extent than hypochlorous acid, thus killing *E. coli* by destroying the cell's outer layer. Compared to the various methods that have been used to evaluate disinfection, the method we used provides a better means to localize cell damage. It is possible to control where *E. coli* expresses the fluorescent protein through transformation with plasmid DNA. Therefore, the damage mechanism can be further elucidated by performing expression in other parts of *E. coli*. Moreover, fluorescence is a clear indicator that makes it possible to examine the amount of fluorescing material released outside the cell, as well as the amount remaining inside. However, fluorescence does not necessarily indicate the amount of leaked EGFP, and measurements depend on the fluorescence intensity of the leaked EGFP. Therefore, this method is a weak tool for quantitative work. Here, we compensated by calculating

the reduction ratio of fluorescence corresponding to each CT value. For such a task, more efficient methods need to be investigated. Nevertheless, it was possible to analyze the mechanism of damage by chlorination on *E. coli* by expressing a fluorescent protein in the *E. coli* cytoplasm, treating it with chlorination, and measuring the amount of EGFP leaked. It is still necessary to clarify the details of the disinfection mechanism of chlorine through further applications of this method.

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