Note

The Effect of Repeated Sodium Hypochlorite Exposure on Chlorine Resistance Development in *Escherichia coli* O157:H7

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Chlorine water has been widely used to reduce viable bacterial contamination on food surfaces and sanitize processing facilities. The emergence and spread of microorganisms with reduced susceptibility to antimicrobial agents is a major public health problem. This *in vitro* study was designed to evaluate the possibility that *E. coli* O157:H7 can acquire chlorine resistance and subsequent induction of cross-resistance to chlorine water. In addition, the occurrence of spontaneous chlorine resistance mutations or a higher mutation rate introduced by chemical mutations as compared to the standard was also examined. Comparisons of bacterial viability, cellular activity and susceptibility to antibiotics before and after repeated cycles of enrichment cultivation were performed. There were no significant changes observed in bactericidal sensitivity during repeated cultivation in both NTG treated and non-treated cells ($n = 6$, $P > 0.05$). There were no significant differences in the activity of cellular respiration enzymes, oxidative stress related gene expression and susceptibility to antibiotics observed in both the NaClO treated samples ($n=8$, $P<0.01$) as compared to the control. These results suggested that it might be difficult for *E. coli* O157:H7 to acquire resistance to chlorine solution even though the bacteria were exposed to NaClO and continued to proliferate.

Keywords: *E. coli* O157:H7, sodium hydrochloride, resistance

Introduction

Chlorine water has been widely used to reduce viable bacteria on food surfaces and sanitize processing facilities (Wei *et al.*, 1985). In aqueous solutions, there is a pH- and temperature-dependant chemical equilibrium between non-ionized hypochlorous acid (HOCl) and hypochlorite ions (ClO⁻). The bacterial cell membrane is permeable to hypochlorous acid, which has a higher molecular oxidative strength than its ionic form (Albrich and Hurst, 1982). The high oxidative activity of hypochlorous acid is thought to damage the cell membrane, leading to bacterial inactivation (Virto *et al.*, 2005).

The emergence and spread of microorganisms with re-

duced susceptibility to antimicrobial agents is a major public health problem. The possibility of drug resistance acquisition is a concern not only in the area of medical treatment but also in relation to food safety issues (Acar and Moulin, 2006). Most bacteria are not believed to acquire genetic resistance to industrial antimicrobial agents such as the bisphenol compound, triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether) (Aiello *et al.*, 2004). However, *Escherichia coli* and *Listeria monocytogenes* were shown to acquire resistance to quaternary ammonium compounds (i.e. benzalkonium chloride) via alteration in the membrane lipopolysaccharide composition and active efflux (To *et al.*, 2002; Lanqsrud *et al.*, 2004).

There is no evidence, based on surveillance by Japanese food processors with a historic use of chlorine water for sanitation purposes, of difficulties in controlling environmental coliform bacteria (*Erwinia* spp., *Citrobacter* spp., *Klebsiella*

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spp., *E. coli*) or spoilage lactic acid bacteria (*Lactobacilli*, *Leuconostoc* spp., Enterococci) (Miura and Kimura, 2006). Therefore, we carried out an *in vitro* study to evaluate the possibility of *E. coli* O157:H7 acquiring chlorine resistance to sodium hypochlorite solution.

Materials and Methods

Bacterial strains and culture conditions *Escherichia coli* O157:H7 CR-3 (isolated from calf feces) was grown in 5 mL of brain heart infusion (BHI) broth (Nissui Co., Tokyo) at 37°C for 16 hr. Cells were collected by centrifugation (3,000 rpm × 10 min) and washed twice with phosphate buffer saline (PBS: 0.3 mM KH₂PO₄, 8.5 g/L NaCl, pH 7.0 by NaOH). To introduce random mutations, PBS-washed cells were treated with 50, 100 and 200 mg/L of N-methyl-N'-nitro-N-nitrosoguanidine (NTG, Wako Pure Chemical Co., Tokyo) for 10 min at room temperature, according to the method of Adelberg *et al.*, 1965. Nutrient agar (Nissui Co., Tokyo) medium was used for viable cell enumeration.

Chlorine resistance challenge test Wild type or NTG treated *E. coli* CR-3 was grown in 40 mL of BHI broth at 30°C for 16 hr. The early stationary growth cells were collected by washing with PBS twice and exposed to 0.1 g/L (as free chlorine) of sodium hypochlorite (Wako Pure Chemical Co., Tokyo) for 5 min at 25°C. As a control, PBS was used as a substitute for chlorine water. Free chlorine was neutralized by the addition of 0.2 g/L of sterile sodium thiosulfate (Saby *et al.*, 1999). The concentration of residual free chlorine was assayed to be less than the detectable limit. The cells were then washed twice with PBS and resuspended in the same volume of PBS. The total number of injured and intact cells was enumerated by plating on Trypticase-soy (TSA) agar (Nissui Co., Tokyo) and Sorbitol MacConkey (SMAC, Nissui Co., Tokyo) agar containing 30 g/L NaCl, respectively. The latter enumeration medium was chosen to suppress the growth of injured cells. Colonies were counted after 24 h of incubation at 37°C. A portion of PBS diluted washed cell culture (1 mL) was inoculated into new BHI medium (50 mL) and incubated at 37°C for 18 h to determine the presence of chlorine resistant mutant strains.

The value representing the effectiveness of chlorine treatment (log reduction) was calculated using the following formula:

Log reduction of viable bacteria = log₁₀ (Viable cell counts after PBS treatment) – log₁₀ (Viable cell counts after chlorine water treatment)

All experiments were repeated at least three times using two cultures for each treatment. Williams' multiple range tests (Williams, 1972) were used for statistical analysis.

Determination of cellular activity Cellular metabolic activity of the culture before and after eight cycles of enrichment cultivation was measured by the ATP chemiluminescent (Lisle *et al.*, 1998; Lisle *et al.*, 1999; Kumar *et al.*, 2007) and menadione-catalyzed luminol chemiluminescent methods (Yamashoji and Takeda, 2001; Kawasaki *et al.*, 2004). PBS washed cultures of non-cycled cultivation and/or cultivated eight times were used for the assays. Cellular ATP level was measured by using a luciferase HS kit (Kikkoman Co., Tokyo). Menadione-catalyzed luminol chemiluminescence was measured using a BactoLumix kit (Nikken Biological-Medical Research Institute Co., Kyoto). The photon counting machine Luminescencer-JNR II AB2300 (ATTO Co., Tokyo) was used for both experiments. The logarithmic values of integrated light emission in 2 s were used as an indicator of cellular metabolic activity. Cultureable bacteria were assayed by the plating method described above. All experiments were performed using two cultures for each treatment and repeated at least three times. Tukey's test was used for analysis of variance.

Determination of chlorine adapted *E. coli* O157:H7 sensitivity to antibiotics Culture (or control culture) cycled eight times was streaked evenly, at a 60° angle, onto the surface of Mueller Hinton agar plates (5 × 40 cm) with a sterile cotton swab. Surplus suspension was removed from the swab by rotating against the side of the tube before the plate was seeded. After the inocula dried, antibiotic impregnated paper discs (SN disk, Nissui) were placed on the agar forceps and gently pressed down to ensure contact. Plates were kept at 15°C for 30 min for better absorption. During this time, microorganisms will not grow significantly but absorption of the antibiotic is allowed to take place. Negative controls were prepared using PBS and reference antibiotics (Streptomycin and Gentamycin) were used as positive controls.

Duplicate inoculated plates containing eight impregnated discs were incubated in an upright position at 37°C overnight and/or 24 to 48 h (if necessary). The results were expressed as the average value of the inhibition zone diameter of the paper disk (8 mm) measured from two orthogonal positions. All experiments were performed twice for each of 4 different series of enrichment studies (n=8). Comparison of the effectiveness of each antibiotic between the strains before and after enrichment cultivation was performed using Student's t-test.

Quantative analysis of stress responses to chlorine

Total RNA extraction The enzymatic lysis protocol for stabilization and isolation of total RNA from bacterial cultures (RNAprotect Bacteria Reagent handbook, Qiagen, July 2001) was followed. Thereafter, the RNA extraction procedure described in the RNAprotect Bacteria Reagent

handbook was followed, using the RNeasy miniprotocol for isolation of total RNA from bacteria. Total RNA was eluted in nuclease-free water and the total RNA concentration was adjusted to 100 ng/ μ l and used for quantitative SYBR green reverse transcription (RT)-PCR assays.

Quantitative SYBR green RT-PCR assay for *micF*, *soxS* and *grpE* mRNA expression The RT reaction and quantitative SYBR green PCR assay was performed using TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA). The RT reaction was done in a separate PCR tube containing 200 μ g of extracted sample RNA, TaqMan RT buffer, 2.5 μ M deoxynucleoside triphosphates, 5.0 mM magnesium chloride, 4 U of RNase inhibitor, 2.5 μ M of random hexamers and 12.5 U MultiScribe reverse transcriptase in a total of volume 10 μ l. After incubation for 30 min at 48°C the cDNA mixture was used as target cDNA in the following SYBR green PCR assay.

The mixture for the SYBR green PCR assay contained 2 μ l of cDNA mixture, TaqMan SYBR green buffer, 200 μ M of dNTPs, 3.0 mM magnesium chloride, 200 μ M of each primer, and 1.25 U of AmpliTaq Gold DNA polymerase in a total of volume of 50 μ l. Thermal cycling was performed with a two-step PCR protocol: 95°C for 10 min, and 35 cycles of 95°C for 20s and 60°C for 1 min.

All RT reactions and PCR amplifications were performed with an ABI PRISM 7900 sequence detector (Applied Bio-

systems), and fluorescence analysis were performed with SDS 2.1 software according to the manufacturer's instructions.

Results

To increase the possibility of the occurrence of spontaneous hypochlorous acid resistant mutants, chemical mutation was introduced. Increasing concentrations of NTG significantly reduced the number of viable cells immediately after treatment. Irrespective of NTG concentration, no significant differences in viable cells were observed after recovery growth (data not shown).

The reduction in viable cell counts of the wild type (NTG untreated) strain after the first NaClO exposure was 4.6 ± 0.9 log CFU/mL on TSA and 4.8 ± 1.0 log CFU/mL on SMAC containing 3% NaCl (Fig. 1a). The reduction in viable cell counts of the NTG treated strain after the first NaClO exposure was 5.1 ± 0.6 log CFU/mL on TSA and 5.0 ± 0.4 log CFU/mL on SMAC containing 3% NaCl (Fig. 1b). If the occasional hypochlorous acid resistant mutant appears in the population of *E. coli* O157:H7, it might proliferate during enrichment cultivation. However, no such change was observed during repeated cultivation in both NTG treated and non-treated cells ($n = 6$, $P > 0.05$).

The reduction in viable bacterial cells (APC) and cellular activity (menadione-catalyzed luminol chemiluminescence

Fig. 1a. Change in the effectiveness of sodium hypochlorite during enrichment cultivation without chemical mutagenesis.

Native *E. coli* O157:H7 CR-3 was treated with NaClO or PBS and grown in BHI broth repeatedly. The circles and triangles represent TSA and SMAC + 3% NaCl as enumeration media, respectively. Closed and open symbols represent PBS (control) and 0.1 g/L (as free chlorine) NaClO treated samples, respectively. The observed differences in these two values correspond to the chlorine sensitivity of the cells.

Fig. 1b. Change in the effectiveness of sodium hypochlorite during enrichment cultivation with chemical mutagenesis.

Chemically-mutated *E. coli* O157:H7 CR-3 was treated with NaClO or PBS and grown in BHI broth repeatedly. The circles and triangles represent TSA and SMAC + 3% NaCl as enumeration media, respectively. Closed and open symbols represent PBS (control) and 0.1 g/L (as free chlorine) NaClO treated samples, respectively. The observed differences in these two values correspond to the chlorine sensitivity of the cells.

Fig. 2. Differences in cellular activity among the cells.

Viable cells (APC) enumerated on two kinds of media (TSA and SMAC), menadione-catalyzed luminol chemiluminescence (CL) and cellular ATP (ATP) levels for the first (NaClO-1) and last (NaClO-8) cycles of enrichment cultivation and first cycle control (PBS-1) are shown [Consider changing the graph so that cell counts are on the left y-axis and CL and ATP levels are on the right y-axis.].

and ATP level) by NaClO treatment was determined and is presented in Fig. 2. A significant reduction in chemiluminescence intensity and ATP was observed in both the NaClO treated samples ($n = 8$, $P < 0.01$) as compared to the control

(PBS treated sample of the first cycle of enrichment cultivation). This reduction is an indicator that cellular activity corresponds to the reduction in viable cell counts. In contrast, no significant differences in the sensitivity to the NaClO

Table 1. Susceptibility of *Escherichia coli* O157:H7 to Ampicillin (ABPC), Ciprofloxacin (CFX), Latamoxef (LMOX), Aztreonam (AZT), Imipenem (IPM), Fosfomycin (FOM), Nalidixic acid (NA), Tetracycline (TC), Streptomycin (SM), and Gentamycin (GM) before and after 8 cultivation cycles with 100 mg/L NaClO.

Antibiotics	Weight in a paper disks (μg)	Diameter of diffusion zone (mm)	
		Before enrichment cultivation (mm)	After enrichment cultivation (mm)
ABPC	10	20.0 $\pm$ 0.4	19.6 $\pm$ 0.8
CFX	30	25.3 $\pm$ 0.5	25.3 $\pm$ 0.8
LMOX	30	27.2 $\pm$ 0.5	26.7 $\pm$ 0.4
AZT	30	28.2 $\pm$ 0.4	28.6 $\pm$ 0.3
IPM	10	30.4 $\pm$ 1.3	30.1 $\pm$ 0.5
FOM	50	25.6 $\pm$ 0.6	25.6 $\pm$ 0.5
NA	30	22.4 $\pm$ 0.4	22.9 $\pm$ 0.6
TC	30	25.9 $\pm$ 0.4	26.1 $\pm$ 0.2
SM	10	16.1 $\pm$ 0.4	15.9 $\pm$ 0.3
GM	10	19.0 $\pm$ 1.0	19.1 $\pm$ 0.5

treatment between the samples of the first and last enrichment cycles were observed ($n = 8$, $P > 0.05$). The reduction in menadione-catalyzed luminol chemiluminescence (CL) by NaClO treatment was 2.1 ± 0.2 log counts/2 s for the first cycle and 2.0 ± 0.1 log counts/2 s for the last cycle sample. The reduction in ATP level by NaClO treatment was recorded as 2.5 ± 0.2 log RLU for the first cycle and 2.7 ± 0.3 log RLU for the last cycle sample. Furthermore, the antibiotic bioassay test was performed assuming that resistance to chlorine would affect resistance to antibiotics. However, no significant differences in the zone of inhibition observed in both the NaClO treated samples ($n = 8$, $P < 0.01$) compared to the control (PBS treated sample of the first cycle of enrichment cultivation) were observed (Table 1).

To evaluate the response of *E. coli* transcriptional regulation genes after chlorine treatment, quantitative SYBR green RT-PCR assay for *micF* (RNA regulator of *ompF*), *soxS* (superoxide response regulon) and *grpE* (encoding a heat shock protein) was performed and the results revealed no significant differences in the amount of mRNA transcription between PBS and NaClO treated cells (data not shown).

Discussion

Microorganisms can acquire drug resistance via several mechanisms, such as changes in the efficacy of efflux pumps, modification of the cellular target, membrane permeability alterations, and antibiotic degradation (Dever and Dermody, 1991; Tenover, 2006). This phenomenon occurs by spontaneous mutation in the corresponding gene on the chromosome or through introduction of drug resistance genes from outside of the cell. The mutant strains tend to be concentrated under selective pressure, typically under low levels of drugs.

In addition, the transcriptional alternation of several stress related genes contribute to adaptation to the drug in existing environments (Giuliodori *et al.*, 2007). However, in our experiment there were no significant differences observed in the expression of *micF*, *soxS* and *grpE* genes, which are reportedly activated by exposure to hypochlorous acid (Duncan *et al.*, 1996).

Since chlorine water is widely used in the sanitation of food facilities and disinfection of raw foods, the ability of pathogenic bacteria to acquire resistance to hypochlorous acid would lead to severe problems. Compared to “target-specific” antibiotic agents, it has been considered difficult for a bacterium to acquire resistance to hypochlorous acid, as the compound is thought to be a nonselective oxidant, reacting readily with a variety of subcellular compounds, such as protein, lipid and nucleic acid. The adaptation to HOCl may be related to the lower levels of cellular damage caused by hydroxyl radicals and singlet oxygen (Mokgatla *et al.*, 2002). However, neither significant increases in chlorine resistance nor any significant changes in cellular responses (including the expression of oxidative stress response genes), before and after repeated exposure to chlorine water, was found in this study. These results suggest that it might be difficult for *E. coli* O157:H7 to acquire genetic resistance to chlorine solution despite bacterial exposure to NaClO and repeated proliferation.

The possibility of chlorine-resistant *E. coli* reflects the ability of the bacteria to evolve such phenotypes. To confirm this possibility we have performed a number of experiments:

- (1) the effectiveness of Brain heart infusion (BHI) medium, M9 synthetic medium and (nutrient rich) trypticase-soy broth on growth and regeneration,
- (2) the effectiveness of chlorine water on the different (“mid-log”, “late-log” and “early stationary”) growth phases of *E. coli* O157:H7 CR-3,
- (3) the effects of various concentrations of active chlorine (50, 100 and 200 mg/L) on *E. coli* O157:H7,
- (4) comparison of the responses in different *E. coli* strains (K12 W3110 and O157:H7 CR-3),
- (5) the optimization of enumeration agar media, to distinguish between intact and injured cells, using several major media for coliforms (VRBA, Desoxycholate agar, Nutrient agar, etc.) supplemented with different concentrations of sodium chloride (0 to 5 %) and,
- (6) the optimum inoculum concentration for repeated cultivation (0 to 4 log CFU/mL as injured cells).

Adaptation to certain experimental conditions (2 and 3) gave inconclusive results due to higher bactericidal effectiveness. However, most of the experimental conditions (1) to (4) did not make any major differences to the conclusion

(Data not shown). Two kinds of enumeration media (TSA and SMAC + NaCl) were chosen based on the experimental results (5) to distinguish the total cells (grown in TSA) and intact cells only (grown in SMAC + NaCl).

Although the formation of bacterial biofilms has been shown to impart higher tolerance to sanitizers (Ryu *et al.*, 2005, Pan *et al.*, 2006), the results described above were generated from an exposure study in a liquid medium, indicating that it is not possible to form biofilms in liquid medium. However, to prevent the formation of bacterial biofilms, an adequate level of precaution is necessary in the cleaning and sanitizing of food processing facilities. In the practical realization of this objective, the establishment of food hygiene training and standard sanitation operation programs (Vaz *et al.*, 2005) is required to ensure product safety.

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