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Research Article

Effect of environmental stresses on the survival and cytotoxicity of Shiga toxin-producing *Escherichia coli*†

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Abstract

The objective of this study was to determine the effect of environmental stresses on the cytotoxicity of Shiga toxin-producing *Escherichia coli* (STEC). STEC O157:H7 and six non-O157 STEC strains (O26:H11, O103:H1, O104:H4, O111:NM, O121:NM, and O145:NM) were subjected to osmotic (a_w 0.95–0.98), acid (pH 4–7), and chlorine (1–5 ppm) stresses. After stress treatments, bacterial populations, expression of virulence-associated genes, and Vero-cytotoxicity were determined. Among the strains, O145:NM survived at a_w 0.97 longer than other serotypes, while O111:NM was significantly more sensitive to osmotic stress. At pH 4, O103:H1 was more resistant to the stress, while O26:H11 and O111:NM had significantly less growth. For 2 ppm chlorine stress, O26:H11, O103:H1, and O145:NM had higher populations (>3 log) than other strains. Stressed strains showed a significant increase in relative gene expression levels of *stx1*, *stx2*, and *eae* in O103:H1, O104:H4, and O145:NM than non-stressed control. Additionally, significantly higher Vero-cytotoxicity, as indicated by lactate dehydrogenase assay, of stressed O26:H11, O103:H1, O104:H4, and O145:NM was observed. The results suggest that the growth and cytotoxicity of selected pathogenic *E. coli* may be enhanced after being exposed to environmental stresses.

Key words: Pathogenic *Escherichia coli*, stress, growth, virulence genes, Vero-cytotoxicity.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) cause severe foodborne illnesses, with patient outcomes including bloody diarrhoea, haemolytic uraemic syndrome, and death. Among serotypes of STEC, *E. coli* O157:H7 draws the major attention because it has been implicated in more foodborne illness outbreaks than other serotypes. In

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recent years, increasing awareness of non-O157 STEC as causes of human illness has prompted expanded clinical diagnosis, investigation, and surveillance of these organisms (Gould *et al*., 2013). In addition, more than 380 other serotypes of STEC have been isolated from humans (Karmali *et al*., 2010). Six *E. coli* O groups comprising 13 serotypes have been known to be responsible for over 70% of non-O157 STEC infections in the USA in the period between 1983 and 2002 (Brooks *et al*., 2005). Strains of non-O157 STEC cause illnesses similar to those caused by O157 STEC, and the virulence is associated with the presence of virulence genes such as *stx*1 or *stx*2 (Shiga toxin), *eae* (intimin), *hly* (enterohemolysin), *nle* [non-locus of enterocyte effacement (LEE)-encoded effector protein], *saa* (STEC auto-agglutinating adhesion), and *subAB* (subtilase cytotoxin) (Paton et al., 2004; Mingle *et al*., 2012). These virulence gene profiles have been successfully revealed by real-time polymerase chain reaction (RT-PCR) (Monaghan *et al*., 2011). Of the several virulence factors involved in pathogenicity, Shiga toxin plays a major role in causing severe illnesses in humans (Thorpe, 2004). The Stx family is comprised of Stx1 and Stx2. Stx1 is highly homogeneous, while Stx2 has several variants (Ju *et al*., 2012). Both Stx1 and Stx2 are enterotoxic in the intestinal segment model leading to death in mice and rabbits and are cytotoxic to various cell culture models. Cytotoxicity assays have been developed to measure the viability of mammalian cells based on cell proliferation or death. One parameter in evaluating cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells. Vero cell cytotoxicity assay has been used for the diagnosis of STEC infection (Paton and Paton, 2003). Vero cells have a high concentration of Gb3 receptors on their plasma membranes as well as Gb4 (the preferred receptor for Stx2e) and thus are highly sensitive to all known Stx variants.

Food manufacturing process is designed to interfere with bacterial homeostasis through inactivation of the bacteria by exposure to adverse conditions. It has been known that STEC responds to sublethal environmental stresses (Montet *et al*., 2009). A number of sublethal processing stresses that bacteria may experience during food processing including osmotic stress, mildly acidic environment, and chlorine treatment may enable their survival at the subsequent processing and adverse environmental conditions and upregulation of the virulence-associated genes (Williams et al., 2000; House *et al*., 2009). In other words, bacteria recovering or recovered from sub-lethal stresses may exhibit altered expression of virulence-associated genes as a result of injury repair (Price et al., 2000; Olesen and Jespersen, 2010).

Sodium hypochlorite (NaOCl) dissolved in water is widely used in the food industry to sanitize equipment and reduce microbial contamination of fresh fruits and vegetables (Erkmen, 2010; Althaus *et al*., 2012). Microorganisms previously exposed to chlorine treatment may survive and be ingested by consumers. Based on recent whole-genome microarrays for *E. coli* O157:H7 strains linked to two foodborne illness outbreaks caused by fresh produce—Sakai, a clinical isolate from the 1996 sprout outbreak in Japan (Michino *et al*., 1999), and TW14359, an isolate from the 2006 spinach outbreak in the USA (Centers for Disease Control and Prevention, 2006)—a total of 240 genes in Sakai and 140 genes in TW14359 were found to be differentially regulated (Deng *et al*., 2011). Despite several transcriptomic analyses, none of the studies revealed that sub-lethal chlorine stress might alter the virulence of pathogenic *E. coli*. Moreover, although non-O157 STEC have been increasingly identified as clinically significant, there is a lack of information as to how non-O157 STEC responds to stress and the effect of stress on the cytotoxicity, which may be an indication of the levels of Shiga-toxin production and virulence. The purpose of this study was to address if there is any quantitative change in Shiga toxin production by non-O157 STEC under specified stress conditions as determined by Vero-cytotoxicity assays.

Materials and Methods

STEC strains

Six *E. coli* non-O157 strains—O26:H11 (05-6544), O103:H1 (04/62), O104:H4 (2009EL-2050), O111:NM (98–8338), O121:NM (03-406), and O145:NM (03-4699)—were kindly provided by Dr. Pina Fratamico (U.S. Department of Agriculture, Agricultural Research Services, Wyndmoor, PA). *E. coli* O157:H7 strains of ATCC 43888, 43889, and 43890 were used as a cocktail mix. Each strain was grown overnight in brain heart infusion broth (BHI; BD Biosciences, Sparks, MD) at 37°C on a shaker with shaking at 200 rpm. Cells were harvested at late logarithmic growth (~10^8 CFU/ml).

Osmotic stress

Stock solutions of various water activities were prepared fresh on the day of the experiment by using 8.5, 7, 5.25, and 3.5 g of NaCl (Mallinckrodt Baker, Phillipsburg, NJ) to obtain different levels of water activities (a_w 0.95, 0.96, 0.97, and 0.98) in 100 ml of tryptic soy broth (TSB; BD Biosciences). Water activity was measured using an AquaLab water activity metre (Decagon Devices, Pullman, WA) calibrated with saturated salt solutions of known water activity. Ten millilitres of the overnight *E. coli* cell suspension cultures was pelleted (2800 × *g*, 2 min, 4°C), washed twice with phosphate-buffered saline (PBS), and diluted in sterile distilled water (DW) to a final concentration of ~10^3 CFU/ml. Samples were incubated at 22°C and samplings were performed after incubation for 7, 14, and 21 days. At each sampling point, 1 ml of cell suspension was removed and serially diluted in PBS. The cell counts were determined by plating 0.1 ml of solution onto tryptic soy agar (TSA; BD Biosciences) plates. The plates were incubated at 37°C for 24–48 h, and the typical colonies were counted.

Acidic stress

TSB was acidified to pH 7, 6, 5, or 4 using 1 N hydrochloric acid (Fisher Scientific, Fair Lawn, NJ). The acidified TSB was sterilized using 0.22-µm Corning filter units (Costar, Corning, NY). *E. coli* cells were inoculated separately into each of the prepared acidified PBS solutions (10 ml) to achieve a final cell concentration of ca. 10^3 CFU/ml. The inoculated broth was incubated at 22°C for 21 d. Samples were drawn after incubation for 7, 14, and 21 days. At each sampling point, 1 ml of each cell suspension was serially diluted in PBS. Three appropriate dilutions (0.1 ml) of each tested sample were plated on duplicate TSA plates. The inoculated plates were incubated for 24–48 h at 37°C before colonies were counted.

Chlorine stress

Stock solutions of NaOCl were prepared fresh on the day of the experiment by diluting 5% NaOCl (Fisher Scientific) in sterile distilled water (22°C) to give various concentrations of NaOCl. Each strain of STEC at 8 log CFU/ml was exposed to NaOCl (1, 2, and 5 ppm) at 22°C for 24 h. Ten millilitres of STEC cell suspension cultures grown overnight at 37°C was pelleted (2800 × *g*, 2 min, 4°C), washed twice with PBS, and diluted in chlorinated distilled water to a final concentration of ~10^7 CFU/ml. During incubation in chlorinated distilled water for 24 h, 1 ml of liquid from each tube was immediately transferred to test tubes containing PBS and filter-sterilized neutralizing reagent (2 µg/ml of sodium thiosulfate, Fisher Scientific) at each sampling period (1, 6, and 24 h). The cells after

the treatments were enumerated by plating 0.1 ml of solution onto TSA agar plates and incubating at 37°C for 24 h. The STEC cells surviving the exposure of 2 ppm chlorine for 24 h were collected and subjected to chlorine exposure treatment for 24 h. After four consecutive exposures, the fourth generation of each chlorine-stressed STEC cells (G_3) and each generation (G_0, G_1, G_2, and G_3) was stored and frozen until they were used for cytotoxicity assays.

Quantification of virulence-associated genes

One millilitre of the bacterial cells grown overnight at 37°C was harvested by centrifugation at 17 900 × *g* for 3 min. After removal of the supernatant, the pellet was subjected to bacterial RNA isolation using RiboPure™-Bacteria Kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. RNA was eluted using TE buffer (10 mM Tris–HCl, 1 mM EDTA at pH 8). The concentration of RNA was evaluated by the absorbance readings at 260 and 280 nm using the Nanodrop® ND100 UV–Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). DNase I was treated according to the manufacturer's instructions prior to cDNA synthesis to avoid residual DNA contamination.

RNA (2 µg) was subsequently used to synthesize cDNA using the SuperScript III First Strand Synthesis kit as recommended by the manufacturer (Invitrogen, Carlsbad, CA). The cDNA (10 ng) was added to 25 µl PCR reaction mixture containing 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and 250 nM of each primer (*stx*1, *stx*2, *eae*, and *mdh*). PCR for *stx*1, *stx*2, *eae*, and *mdh* genes was carried out using the primer pairs (Fratamico *et al*., 2011) as described in Table 1. PCR samples were amplified using a 7500 Fast RT-PCR Systems (Applied Biosystems) at a cycle of 95°C for 10 min, followed by 35 cycles of 95°C for 15 s, then 55°C for 1 min. Non-DNA templates were included as negative controls for PCR reaction. The housekeeping gene *mdh* was used as an internal control to normalize the *Ct* (threshold cycle) values of *stx1*, *stx2*, and *eae*. The relative expression of these virulence genes in stressed cells was calculated by normalizing to the expression level of corresponding genes in control cells. The ΔCt, $\Delta\Delta Ct$, and the 2^{-fx} values were calculated as described by Pfaffl (2001).

Vero-cytotoxicity assays

Cytotoxicity was determined using Vero cells (ATCC CCL-81, African green monkey kidney epithelial cell line). Lactate dehydrogenase (LDH) assay was used to quantify cell death based on the measurement of LDH released from the cytosol of damaged cells into the extracellular media. LDH is a stable cytoplasmic enzyme present inside the cytosol of all types of bacterial cells. It is released to the extracellular space when the plasma membrane is damaged (Korzeniewski and Callewaert, 1983). The LDH activity is determined by a two-step enzymatic test. The first step is the reduction of NAD^+ to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In a second step, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, which is reduced to a red formazan (Lappalainen *et al*., 1994). For assays, monolayer of subconfluent Vero cells in 96-well plates was seeded in 96-well plates. STEC cells (G_3 or control) grown overnight under regular BHI broth at late exponential or early stationary phase were collected and filter-sterilized to obtain supernatants including Shiga toxin released to extracellular media. Vero cells were exposed to filter-sterilized supernatants for 24 h or up to 48 h at 37°C in 5% CO_2 incubator. After incubation, the release of LDH was measured using the Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The spontaneous LDH release (=low control) was determined by adding 100 µl of assay medium that contains 1% foetal bovine serum without pyruvate to wells containing 100 µl of cells in assay medium. Maximum LDH release (=high control) was determined by adding 100 µl of Triton X-100 (Sigma, St. Louis, MO) solution (2% Triton X-100 in assay medium) to each well containing 100 µl of cells in assay medium. The amount of LDH released was determined by transferring 100 µl of supernatant from each well to wells of optically clear 96-well, flat-bottomed microplates (Corning), adding 100 µl of reaction mixture to wells, incubating at room temperature for 30 min in the dark, and measuring absorption at 492 nm using a reference wavelength of 620 nm with Saffire2 microplate reader (Tecan, Morrisville, NC). In the cytotoxicity LDH detection assay, the percentage of cytotoxicity was calculated using optical density readings:

$$\text{Cytotoxicity}(\%) = \left[\frac{(\text{experiment value} - \text{low control})}{(\text{high control} - \text{low control})}\right] \times 100$$

Measured cytotoxicity was normalized by the cell population when the supernatants were collected. Each sample was tested in triplicates. At least three independent experiments were performed for each sample.

Table 1. Primer pairs used in this study for qRT-PCR.

Primer	Sequence (5′–3′)	Target gene
stx1-F	GAC TGC AAA GAC GTA TGT AGA TTC G	*stx1*
stx1-R	ATC TAT CCC TCT GAC ATC AAC TGC	
stx2-F	ATT AAC CAC ACC CCA CCG	*stx2*
stx2-R	GTC ATG GAA ACC GTT GTC AC	
eae-F	CTT TGA CGG TAG TTC ACT GGA C	*eae*
eae-R	CAA TGA AGA CGT TAT AGC CCA AC	
mdh-F	AGT ACT CCC GTT TCT CT	*mdh*
mdh-R	CAG GGC GAT ATC TTT CTT GA	

Statistical analysis

Each experiment was conducted in duplicate and repeated three times. Data collected from the study were analysed using the Student's *t*-test of the Statistical Analysis Software (SAS Institute Inc., Cary, NC). Significance of differences in survival among the tested STEC strains under various treatments was determined at $\alpha = 0.05$.

Results and Discussion

Survival under osmotic stress

The populations of three strain cocktails of *E. coli* O157:H7 and six strains of non-O157 STEC (O26:H11, O103:H1, O104:H4, O111:NM, O121:NM, and O145:NM) subjected to four different levels of osmotic stresses (a_w 0.95, 0.96, 0.97, and 0.98) at 22°C for 3 weeks are shown in Figure 1A. Most foods have water activity of 0.95 or above and a_w 0.99 was not included in this experiment because of similar outcome to control in growth. At a_w 0.95 and 0.96, all tested strains fell to undetectable levels with little variation after a week of incubation. At a_w 0.97, 7.6 log CFU/ml of *E. coli* O145:NM strain survived the osmotic stress during 21 days of incubation, while the other strains were not detected after one week. *E. coli* O111:NM was most sensitive to osmotic stress; even at a_w 0.98, it did not endure the osmolarity for 7 days. The other strains showed similar growth for 21 days. Previous studies (Baylis *et al*., 2004; Hiramatsu *et al*., 2005) have shown that *E. coli* O157 and

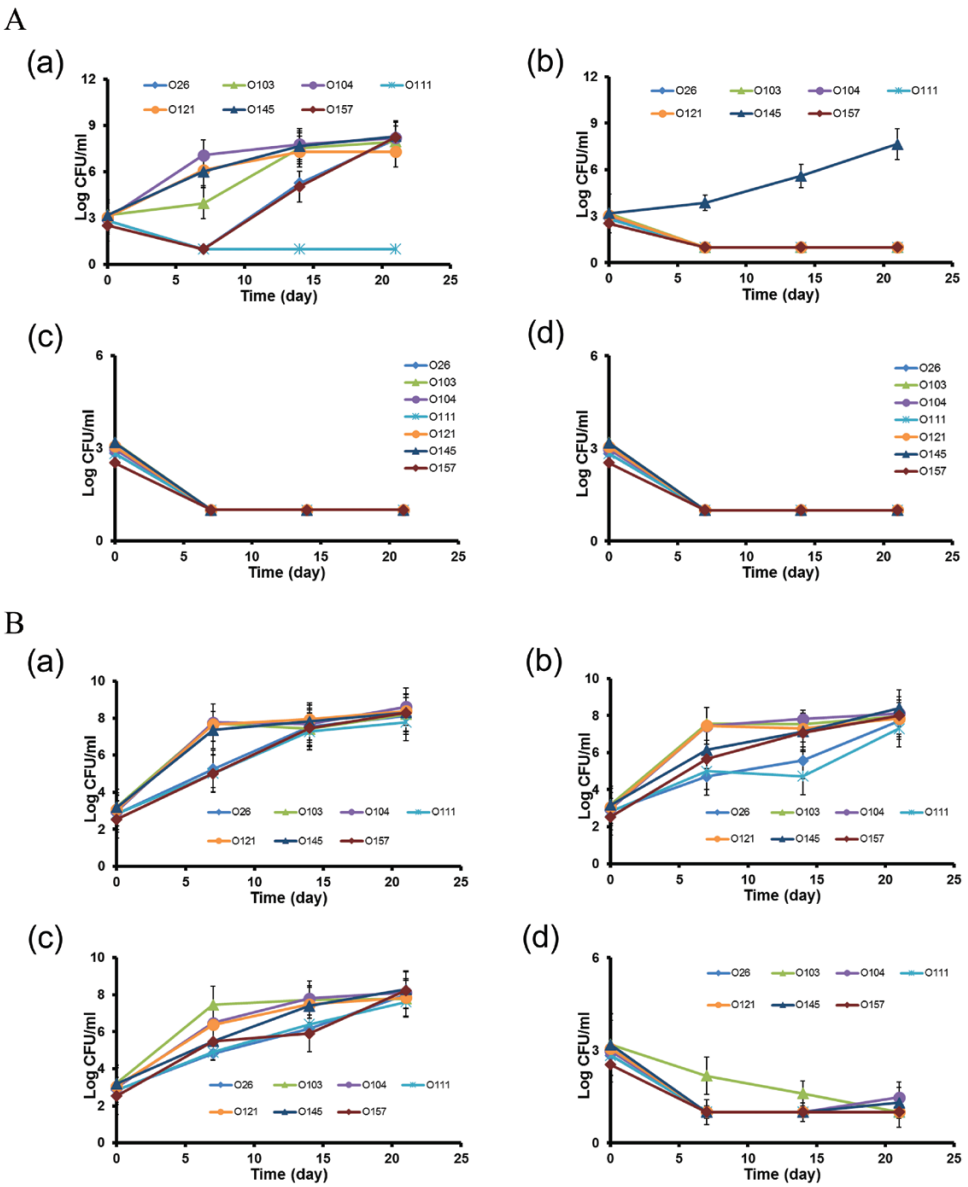

Figure 1. Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) show similar survival patterns to *E. coli* O157 under osmotic and acidic stresses. Survival curves of STEC (O26:H11, O103:H1, O104:H4, O111:NM, O121:NM, O145:NM, and O157:H7) under various (A) osmotic pressure [a_w 0.98 (a), 0.97 (b), 0.96 (c), and 0.95 (d)] and (B) acidic stress [pH 7 (a), 6 (b), 5 (c), and 4 (d)] are plotted. Cells were inoculated at 3 log CFU/ml and incubated at 22°C for 3 weeks. Survived cells were enumerated on tryptic soy agar (TSA) plates at 37°C. Data from three independent experiments are presented as means.

non-O157 STEC strains had similar growth patterns under osmotic stresses. In our study, most of non-O157 and O157 STEC showed similar patterns of growth, which is consistent with previous findings except for *E. coli* O145:NM, which exhibited significantly higher growth at a_w 0.97 when compared with other strains. It was suggested that an increase in environmental osmotic pressure disrupts metabolic activities by losing water to the exterior, which leads to the breakage of STEC cell membrane (Beney *et al*., 2004; Yoo and Chen, 2010).

Survival under acidic stress

Seven STEC strains at initial bacterial populations of 3 log CFU/ml were exposed to four different levels of pH (4, 5, 6, and 7) at 22°C for 3 weeks (Figure 1B). Foods associated with recent outbreaks including tomatoes (pH 4–5), ground beef (pH 5–6), Romaine lettuce (pH 6), and spinach (pH 6–7) were considered to choose different levels of pHs. At pH 4, most strains did not survive after 7 days, while 2.2 log CFU/ml of *E. coli* O103:H1 survived after 7 days and 1.8 log CFU/ml remained after 14 days. Over pH 5, most strains did not show any difficulty in growth, where *E. coli* O26:H11 and *E. coli* O111:NM had significantly ($P < 0.05$) less growth than other strains during the first week. Acidic stress is a stress that STEC may be exposed to in the environment, during food processing, or in food matrices. Typically, different organic acids including benzoate, sorbate, propionate, and citrate are present in combination with mild acidic conditions (pH 4–7). Survival of STEC under the influence of pH was investigated mainly due to the inherent resistance of STEC. Exposed to simulating gastric acidity, *E. coli* O157 was shown to be more resistant to acidic conditions than non-O157 STEC such as *E. coli* O26:H11 and *E. coli* O111:H8 (Bergholz and Whittam, 2007), which is consistent

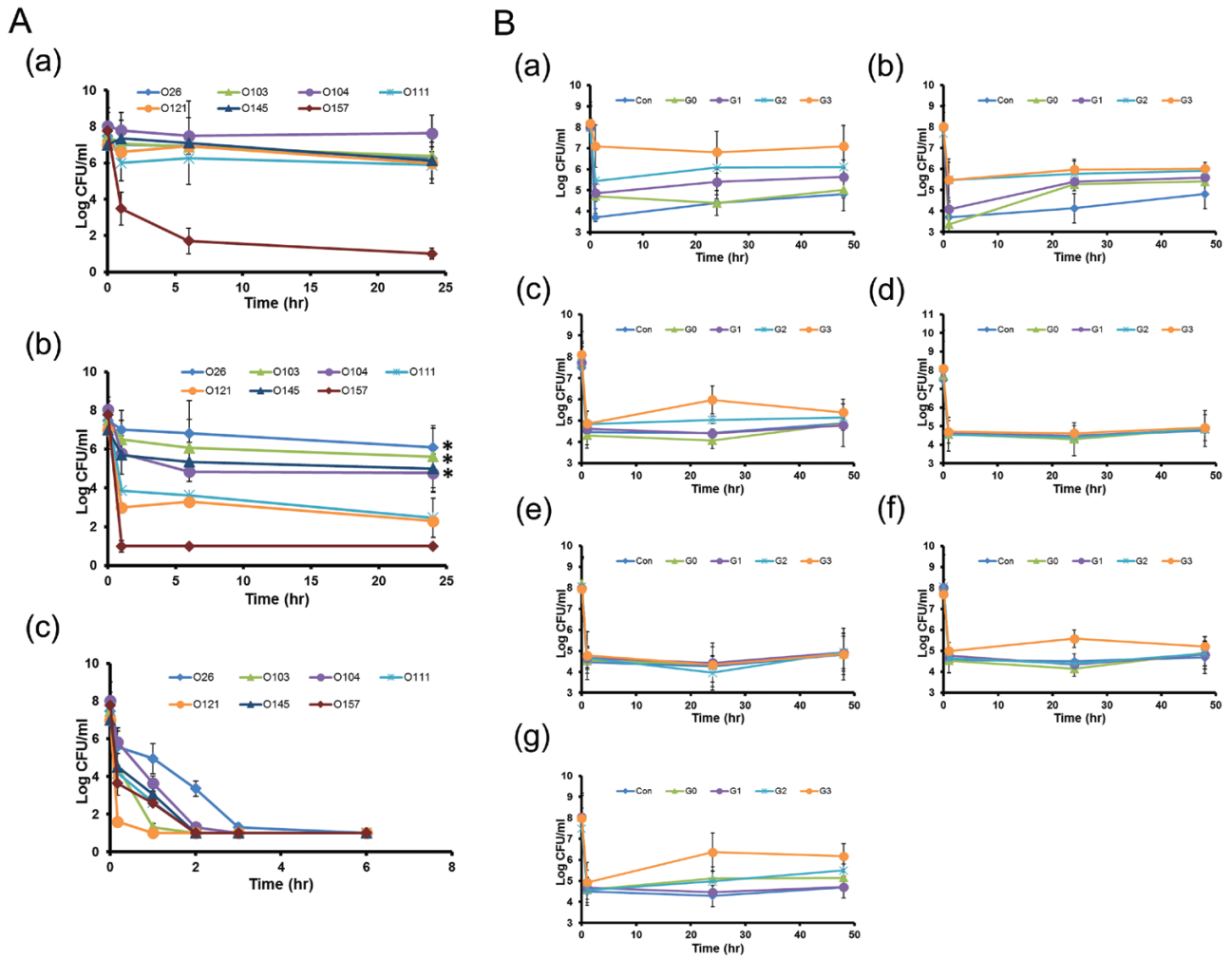

Figure 2. Some of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) are more resistant to chlorine treatment than *E. coli* O157. (A) Sub-lethal concentration of sodium hypochlorite [1 (a), 2 (b), and 5 ppm (c)] was applied to 8 log CFU/ml of STEC grown overnight at 37°C, followed by sampling, neutralization, plating onto TSA plates, and incubation at 37°C until being counted. Data from three independent experiments are presented as means ± SE. *$P < 0.05$. (B) STEC cells [O26 (a), O103 (b), O104 (c), O111 (d), O121 (e), O145 (f), O157 (g)] with acquired resistance to chlorine (G_0, G_1, G_2, and G_3) were subject to chlorine treatment (2 ppm) for prolonged time (48 h). Cells were enumerated as above. The results are the average of three independent experiments.

with our results that these two strains had significantly less growth than other strains at pH 5 in the first week of exposure.

Survival after chlorine stress

After STEC cells at 8 log CFU/ml were exposed to NaOCl (1, 2, and 5 ppm) at 22°C for 24 h, the survivor curve of each strain was plotted and compared using Student's *t*-test (Figure 2A). There were significant differences ($P < 0.05$) in the surviving populations of STEC during chlorine exposures. Significantly higher populations were observed for *E. coli* O26:H11, *E. coli* O103:H1, *E. coli* O104:H4, and *E. coli* O145:NM strains than others including *E. coli* O157:H7 under 2 ppm of NaOCl. The populations of *E. coli* O26:H11 and *E. coli* O103:H1 were 6.1 and 5.6 log CFU/ml, respectively, while other strains were less than 3 log CFU/ml after 24 h. For 1 ppm of chlorine treatment, *E. coli* O104:H4 showed significantly the highest survival among the strains ($P < 0.05$). After 24 h of exposure, cells were washed by PBS, collected, and repeatedly exposed to chlorine. The STEC strains were designated as G_0, G_1, G_2, and G_3 according the number (1–4) of exposure to chlorine stresses. To address the acquired resistance after sub-lethal stress, stressed STEC strains from each generation were exposed to 2 ppm of chlorine for 48 h (Figure 2B). The fourth generation (G_3) of each strain showed significantly ($P < 0.05$) higher survival rate than other generations except for *E. coli* O111: NM and *E. coli* O121:NM, indicating that G_3 is relatively most resistant to sub-lethal chlorine. Activity of chlorine can strictly depend on the matrix where there is other organic material available in the environment. However, we assume that the surface of the equipments in food processing facilities has been taken care of by sanitation and cleaning procedure required by the pre-requisite program accompanying Hazard Analysis Critical Control Point. Some STEC may endure those procedures and remain on the surface where organic matter is absent. Among the intervention strategies to mitigate STEC risk in food production, chlorine is widely used as a washing or sanitizing agent. In this study, the chlorine stress conditions simulate the conditions that the STEC may encounter during food production or in the processing environment. Consecutive exposures of surviving *E. coli* O157:H7 to sub-lethal amounts of chlorine may induce expression of *ycfR*, which is involved in the response to chlorine stress (Hou *et al.*, 2013). It promotes attachment and colonization of *E. coli* O157:H7 in lettuce and leads to

better growth. In other studies, *ycfR* was upregulated by tenfold under chlorine treatment (Zhang et al., 2007; Mermod *et al.*, 2012). Under chlorine stress, this gene has been shown to be involved in the surface hydrophobicity and biofilm formation (Gonzalez-Escobedo and Gunn, 2013). This gene was also known to contribute to surface attachment and survival of *Salmonella enterica* during post-harvest minimal processing of fresh produce (Salazar *et al.*, 2013).

Effect of stresses on the relative expression of virulence-associated genes

Several virulence-associated genes in STEC have been identified (Kaper *et al.*, 2004). Among them, *stx*1, *stx*2, and *eae* have been selected to investigate the effects of chlorine stresses on the relative expression of these genes by RT-PCR. As shown in Figure 3A, consecutive sub-lethal chlorine treatment against O103:H1, O104:H4, O145:NM, and O157:H7 resulted in significant ($P < 0.05$) increase (>1.4 fold) in relative gene expression of *stx*1 compared to control cells. Similarly, chlorine-stressed cells of O103:H1 showed significant ($P < 0.05$) increase (>2 fold) in relative gene expression of *stx*2 compared to control cells, and cells of O104:H4 and O145:NM showed slight increase (1.27–1.38 fold), while O26:H11 showed large increase (1.92 fold) in *stx*2 (Figure 3B). On the other hand, chlorine-stressed cells of O111: NM, O121:NM, and O157:H7 showed similar or slightly less expression of selected genes when compared to control cells. Relative gene expression of *eae* was mostly similar to the patterns in *stx1* and *stx2*, except for stressed cells of O111: NM with twofold increases in *eae* compared to control cells (Figure 3C). Studies have reported that the expression of virulence-associated genes is connected to better survival or growth of STEC in fresh produce. Robinson *et al.* (2006) suggested that the *stx*2 mutant of a STEC strain was less effective in adherence to epithelial cells than its wild-type counterpart. In addition, the mutant revealed a lower capacity to colonize mouse intestine, indicating that Shiga toxin promotes intestinal colonization, possibly also in humans. However, Sheng *et al.* (2006) proposed that *E. coli* O157:H7 requires the presence of intimin, Tir, and pO157 to colonize cattle at the terminus of rectal mucosa, while the colonization preceded normally in the absence of the active *stx* genes (Los *et al.*, 2012). When the pathogen has abrupt shifting in its environment, it induces a package of stress response genes. A recent study (Kocharunchitt *et al.*, 2014) showed that sudden downshift of a_w induced global changes in growth kinetics, nutrient metabolism, cell structure components, and general stress response as well as virulence properties. Changes in relative transcriptional level of virulence-associated genes may be dependent on the general stress response to chlorine stress. Our findings suggest that chlorine stress may induce the expression of virulence-associated genes through potential mechanisms by stress response genes.

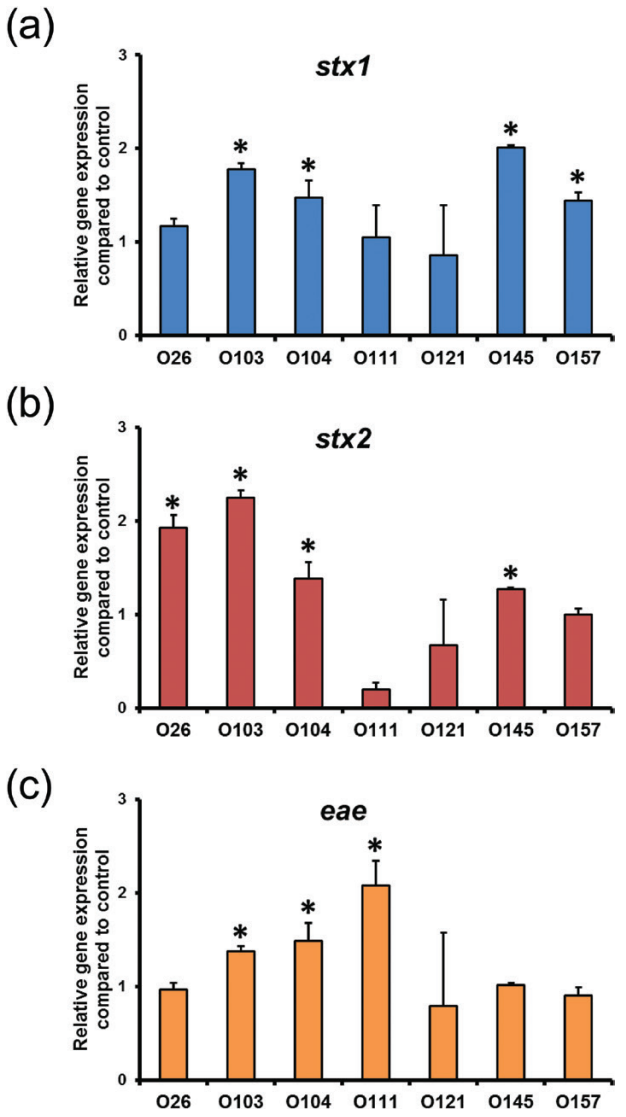

Figure 3. Chlorine treatment enhances expression of virulence genes in Shiga toxin-producing *Escherichia coli* (STEC). Quantitative RT-PCR of STEC was performed to determine the relative levels of *stx1* (A), *stx2* (B), and *eae* (C). *Ct* (threshold cycle) values of *stx1, stx2,* and *eae* were normalized to that of the housekeeping gene, *mdh*. The relative abundance of virulence genes in stressed cells was then calculated by normalizing to the expression level of corresponding genes in control cells. Data from three independent experiments are presented as means ± SE. *$P < 0.05$.

Effect of stresses on the cytotoxicity in Vero cells

Filtered supernatant samples from stressed (G_3) and control STEC cells were tested for the presence of Shiga toxin using the LDH assay. Vero-cytotoxicity increased over time in control and stressed cells, indicating time dependence of toxin production (Figure 4A). The cytotoxicity peaked after 24 h of growth in both stressed and control cells except for *E. coli* O103:H1, with the highest production observed at 36 h for control cells and 48 h for stressed cells during incubation. Therefore, a 24-h growth was chosen for experiments. To address the difference in the cytotoxicity between stressed and control cells, supernatant samples from both groups were collected after 24 h of growth and assayed for Vero-cytotoxicity (Figure 4B). There was significant ($P < 0.05$) increase in cytotoxicity (%) between stressed and control cells in *E. coli* O26:H11 (40%), *E. coli* O103:H1 (50%), *E. coli* O104:H4 (60%), and *E. coli* O145:NM (25%). Stressed *E. coli* O157:H7 cells also showed an increase in cytotoxicity compared to control cells, but this increase was not statistically significant ($P < 0.05$). It was noted that supernatants from stressed and control cells showed an increase in Vero-cytotoxicity over time for the first 24 h. It seems that the damaged cell membrane may enable more toxin release while minimizing the wound of cytosolic organs. An investigation of bacterial recovery from 5-day starvation and chlorine treatment showed that there was no difference between control and stressed STEC in Vero-cytotoxicity (Kolling and Matthews, 2007). The main reason for STECs to produce Shiga toxin might be to protect themselves from either predators or hostile mammalian immunological system (Los *et al.*, 2012). A

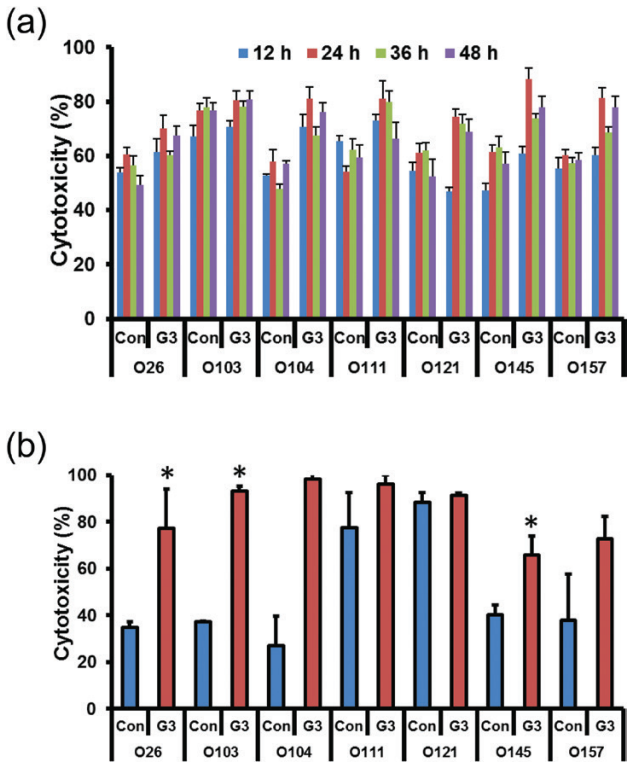

Figure 4. Chlorine stress induces Vero-cytotoxicity of non-O157 Shiga toxin-producing *Escherichia coli* (STEC). Vero-cytotoxicity was assessed using lactase dehydrogenase assay of Vero cells treated with supernatants of STEC cells. (A) Supernatants were collected every 12 h from STEC for 48 h. (B) Supernatants were collected after 24 h of growth. Vero-cytotoxicity was determined and normalized by each cell density population. The results are the average of three independent experiments. $*P < 0.05$.

similar outcome was shown in a study where *Campylobacter jejuni* injured by sub-lethal exposure to chlorine showed a low invasive potential for HeLa cells (Terzieva and McFeters, 1992). In contrast, a virulence study based on mouse LD_{50}, adhesion, and invasion assays conducted in *Yersinia enterocolitica*, *Salmonella typhimurium*, and enterotoxigenic *E. coli* showed that the chlorine treatment weakened their virulence (LeChevallier *et al.*, 1985). Increased release of Shiga toxin production by STEC may benefit the organisms by providing protective barriers to defend themselves against the hosts' immune systems and prolong the life span of invading microorganisms. Transcriptomic responses of *Salmonella enteritidis* and *S. typhimurium* to chlorine stress revealed that half of the genes in *Salmonella* pathogenicity island (SPI) 1 were downregulated in *S. typhimurium* under chlorine treatments, suggesting that chlorine stress may affect the biosynthesis and assembly of type III secretion system leading to reduced virulence in *S. typhimurium*, while no significant change in the expression of genes in SPI was seen in *S. enteritidis* under the same condition (Wang *et al.*, 2010). Additionally, osmotic stress by the use of salt in meat processing enhanced the production of Shiga toxin based on qRT-PCR and Western blot (Harris *et al.*, 2012). Lee *et al.* (2012) suggested that strains which have different genotypes (*stx1*, *stx2*, or both) may respond distinctly to the same stress. They showed that a strain with greater resistance to one type of stress tended to have greater resistance to the other types of stresses. In the present study, the result of higher expression of *stx1* or *stx2* in stressed O26:H11, O103:H1, O104:H4, and O145:NM agrees with their findings. In this study, a cocktail mix of *E. coli* O157:H7 consisting of strain 43888 (lack of *stx1* and *stx2*), 43889 (*stx2*-positive), and 43890 (*stx1*-positive) (Jothikumar and Griffiths, 2002) was used as a control to normalize the relative contribution of each toxin to cytotoxic effect in Vero cell culture. It seemed to have contributed to a larger variation of cytotoxicity in control cells of O157:H7 (Figure 4). The relative abundance of *stx1* and *stx2*, or *eae* in stressed non-O157 STECs of selected strains may enhance the Vero-cytotoxicity in those cells when compared with those of unstressed control cells.

Conclusions

In this study, we compared the level of cytotoxicity between unstressed and stressed STEC. Results suggest that repetitive exposure of STEC to sub-lethal chlorine treatment may enhance the levels of Vero-cytotoxicity in STEC O26:H11, O103:H1, O104:H4, and O145:NM which may lead to an increased virulence. It was supported by the increased expression of virulence-associated genes in STEC O103:H1, O104:H4, and O145:NM. We assume that sub-lethal chlorine treatment may bring about more risks in terms of cytotoxicity. However, to better assess the potential risks, profound biochemical experiments will be needed to sophisticatedly address the factors involved in the release of Shiga toxin and the increase in the expression of virulence-associated genes.

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