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ORIGINAL ARTICLE

Effects of chemical and low-temperature treatments and adaption on the responses of virulence factor genes and outer membrane proteins in *Escherichia coli* O157:H7

Da-Hao Huang [a,d], Kun Wang [b,d], Chihwei P. Chiu [a], Tzu-Ming Pan [c], Tsung-Yu Tsai [a,*]

[a] *Department of Food Science, Fu Jen Catholic University, New Taipei City, Taiwan*
[b] *Division of Gastroenterology, Department of Internal Medicine, Cardinal Tien Hospital, New Taipei City, Taiwan*
[c] *Department of Biochemical Science and Technology, National Taiwan University, Taipei City, Taiwan*

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gene expression of shiga-like toxin II;
reverse transcription multiplex TaqMan polymerase chain reaction;
two-dimensional electrophoresis

Background: In the years after the discovery of this pathogen, *Escherichia coli* O157:H7 has become increasingly prominent, and outbreaks have been reported in many areas.
Methods: In the current study, we determined the expressions of virulent factor genes shiga-like toxin II (*stxII*) and *E. coli* attaching and effacing (*eaeA*), in strains that were isolated from cattle feces and a clinical case in Taiwan. In addition, the effects of chemical and low-temperature stresses and adaptions on the expressions of virulent factor genes were investigated. Furthermore, the outer membrane proteins of acid-adapted *E. coli* O157:H7 TWC01 (TWC01) was separated using two-dimensional electrophoresis, and proteins were identified using mass spectrometry in order to illustrate the changes in protein expression after adaption.
Results: Expressions of *stxII* and *eaeA* in the TWC01 isolated from a clinical case were higher than those in two strains isolated from cattle feces, and both organic and inorganic acid stresses and adaptions enhance the expression of genes encoding virulent factors in strains. In addition, the outer membrane proteins of TWC01 were regulated under hydrochloric acid adaption, indicating induction of acid tolerance and enhancement adhesion in TWC01. Lactic acid treatment of TWC01 resulted in downregulation of channel protein and adherence-related protein expressions.

* Corresponding author. Department of Food Science, Fu Jen Catholic University, Number 510, Zhongzheng Road, Xinzhuang District, 24205 New Taipei City, Taiwan.
E-mail address: tytsai@mail.fju.edu.tw (T.-Y. Tsai).

[d] These authors contributed equally to this work.

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Conclusion: The results of this study are helpful in understanding the resistance of locally isolated TWC01 to chemical and low-temperature stresses, and improving the control of this pathogen.

Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 has become a pathogen of global concern. This strain, first recognized as a human pathogen in 1982, is now known to cause hemorrhagic colitis and hemolytic uremic syndrome.[1] In the years after the discovery of this pathogen, *E. coli* O157:H7 has become increasingly prominent, and outbreaks have been reported in Australia, Canada, Japan, the United States, and various countries in Europe and South Africa.

Sources of *E. coli* O157:H7 infection include ground beef, dairy products, packaged apple juice, drinking water, and municipal sewage.[2] Therefore, *E. coli* O157:H7 is exposed to a variety of environmental stresses and exhibits a stress response to them. This results in resistance to acidic, alkaline, and salty substances.[3–6] Acidity, temperature, pressure, and other physical and chemical methods are commonly used for food processing and preservation. In addition, the use of detergents and bactericides for environmental hygiene also expose microorganisms to extreme conditions. Microorganisms quickly develop adaptation and stress responses to environmental stresses. These adaptations are diverse and unpredictable, creating challenges to food and environmental safety. Therefore, investigating the effects of stresses on microorganisms is an important part of the study of pathogens.

In a previous study, we analyzed the total proteins of acid-adapted *E. coli* O157:H7 using mass spectrometry and determined that most of the proteins were metabolism related, including phosphoglycerate kinase, glutamate decarboxylase α and β, adenine phosphoribosyltransferase, and dihydrodipicolinate synthase. Other proteins were involved in translation and protein folding, and were membrane components.[7] With respect to function, the major difference between the outer and inner membranes is that the outer membrane, because of the presence of pore-forming proteins (porins), is considerably more leaky than the cytoplasmic membrane.[8] In addition, these proteins are also the first cell materials exposed to the environment. We have narrowed our focus to the outer membrane proteins. Due to the concepts of food safety, the effects of food processing and hygiene on this pathogen need to be examined. In the current study, we determined the expressions of virulent factor genes shiga-like toxin II (*stxII*) and *E. coli* attaching and effacing (*eaeA*), in strains that were isolated from cattle feces and a clinical case in Taiwan. In addition, the effects of chemical and low-temperature stresses and adaptions on the expressions of virulent factor genes were investigated. Furthermore, the outer membrane proteins of acid-adapted *E. coli* O157:H7 TWC01 (TWC01) was separated using two-dimensional electrophoresis (2-DE), and proteins were identified using mass spectrometry in order to illustrate the changes in protein expression after adaption.

Methods

Bacterial strains and cultural conditions

Strain TWC01 was isolated from the first clinical case in Taiwan.[9] Strains *E. coli* O157:H7 TWE01 and TWE04 (TWE01 and TWE04) were isolated from cattle feces in northern Taiwan.[10] Prior to testing, the bacterial strains were retrieved from frozen storage and cultured in nutrient broth (NB; Difco Corporation, Sparks, MD, USA) overnight at 37°C. The cultures were then transferred in tryptic soy broth (TSB; Difco Corporation) and incubated at 37°C until the OD_{600} reached 1.2–1.4. Cells were subsequently harvested and used in this study. In addition, they were checked for genotype (*eaeA*, rfb_{O157}, and *stxII*) and the expressions of virulent factor genes by using polymerase chain reaction (PCR)[11] at the 2nd hour, 4th hour, 6th hour, and 8th hour during 24 hours of incubation.

Preparation of chemical and low-temperature stress-adapted cells

Initially, the cells (2.0×10^6 CFU) were stressed with fresh TSB containing 50 ppm sodium hypochlorite (NaClO), 5mM fumaric acid (FA), at pH 6.5 adjusted using hydrochloric acid (HCl) or lactic acid (LA), and incubated at 37°C for 24 hours. Cell growth was determined by measuring the OD_{600} every 1 hour. In addition, cells (2.0×10^6 CFU) were in fresh TSB containing 20% glycerol and stored at 4°C, −30°C, and −196°C for 16 hours to prepare low-temperature-stressed cells, and then cultured at 37°C for further analysis. Gene expressions of these cells, which were stressed with HCl, LA, and low temperature, were analyzed at the 2nd hour (initial log phase), 4th hour (mid log phase), 6th hour (initial stationary phase), and 8th hour (mid stationary phase) during 24 hours of incubation. Moreover, gene expressions of the cells treated with NaClO and FA were determined at the 10th hour (initial log phase), 12th hour (mid log phase), 14th hour (initial stationary phase), and 16th hour (mid stationary phase) during 24 hours of incubation.

Procedures described in a previous paper were followed to prepare the chemical-adapted cells of *E. coli* O157:H7.[7,12,13] Briefly, the cells obtained were centrifuged at $3000 \times g$ for 10 minutes. After resuspending the pellets in phosphate buffer saline, pH 7.2, the cultures were centrifuged at $3000 \times g$ for 10 minutes again. Initially, the pellets were resuspended in fresh TSB containing 50 ppm

NaClO and 0.005M FA, at pH 6.5 adjusted using HCl or LA, and incubated at 37°C for 16 hours. Cell growth was determined by measuring the $O.D._{600}$ every 4 hours. When the growth curves of cells incubated in this condition were the same as those of untreated cells, the treated cells were inoculated in the next treated condition (100 ppm NaClO, 0.01M FA, pH 6.0 using HCl or LA). Finally, the adapted cells needed to be confirmed that adapted 200 ppm NaClO, 0.05M FA, pH 4.0 HCl or LA, respectively, and for further analysis.

Total RNA preparation and reverse transcription real-time PCR using the TaqMan system to quantify expressions of *stxII* and *eaeA*

Total RNA was isolated from *E. coli* O157:H7 by the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) using a silica-gel-based membrane. Cells were harvested by centrifugation and resuspended in Tris-EDTA buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0) containing 400 μg/mL lysozyme for 5 minutes. DNA was completely removed by RNase-free DNase set (Qiagen Inc.) according to the manufacturer's instructions. RNA quantification was carried out by a sensitive fluorescence-based solution assay (RiboGreen RNA quantification reagent; Molecular Probes, Leiden, The Netherlands) following the manufacturer's description.

Procedures and sequences of probes and primers used in reverse transcription real-time PCR with the TaqMan system were described in our previous paper.[14] Briefly, the SuperScript III RT/PlatinumR *Taq* Mix (Invitrogen Co., Carlsbad, CA, USA) was used for the assay in one-step RT TaqMan PCR. DNase-treated RNA samples (500 ng) were amplified with 1 μL SuperScript III RT/PlatinumR *Taq* Mix in 1 × reaction buffer (0.4mM of each deoxynucleoside triphosphate (dNTP) and 6mM $MgSO_4$). Reactions were performed in a total volume of 20 μL containing 0.3 μM of each of the primers, 0.05μM RTeaeP, and 0.1μM RTO157P (determined *eaeA* gene expression) or 0.15μM RTstxIIP and 0.1μM of RTO157P (determined *stxII* gene expression) fluorogenic probes in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Amplification was performed in a two-step thermal program. cDNA was synthesized at 55°C for 15 minutes, heated at 95°C for 2 minutes, and then amplified for 40—45 cycles (denaturing at 95°C for 15 seconds and annealing at 60°C for 30 seconds). Reaction conditions for amplification and the parameters for fluorescence data collection were programmed into a Power Macintosh 4400/20 (Apple Computer, Santa Clara, CA, USA) linked directly to the ABI Prism 7700 sequence detection system using the SDS 1.6 application software. After real-time data acquisition, the cycle threshold value was calculated by determining the point at which fluorescence exceeded an arbitrary threshold signal (10-fold higher than the baseline). The threshold signal was manually set so that it intersected the amplification curves in the linear region of the semilog plot. The assays were carried out in triplicate cultures. In addition, the standard DNA plasmids (P_{stxII}, P_{eaeA}, and $P_{rfbO157}$) constructed in our previous study[14] (10^9 copies), no template control, no amplification control, and no DNA control were included in each run (96 reactions). The relative expressions of *stxII*/rfb_{O157} and *eaeA*/rfb_{O157} were determined.

Sample preparation and two-dimensional gel electrophoresis

The cells that were grown until the initial stationary phase were harvested by using 10,000*g* for 10 minutes at 4°C and washed three times with 10mM Tris-HCl. Then, the cells were resuspended with 7 mL 10mM Tris-HCl (pH 7.0) and disrupted by sonication (Misonix XL-2020; Misonix Inc., Farmingdale, NY, USA) on ice for 2 minutes (power of 85.5—95 W, 10-second pulse). Lysates were centrifuged (7500*g*, 15 minutes, 4°C) to remove unbroken cells. The supernatants were diluted to 60 mL with 0.1M carbonate buffer (pH 11.0) and incubated with stirring at 4°C for overnight. The extracts were recovered by ultracentrifugation (120,000*g*, 1 hour, 4°C), and the pellets were rinsed and solubilized with acetone containing 10% trichloroacetic acid and 0.007% β-mercaptoethanol for 2—4 hours. After centrifugation at 12,000*g* at 4°C for 30 minutes, the pellets were washed three times with acetone containing 0.007% β-mercaptoethanol and dried. Finally, the pellets were solubilized in modified cell lysis buffer [30mM Tris-Cl (pH 8.5), 2M thiourea, 7M urea, and 1% ASB-14] at room temperature for 30—60 minutes. Membrane proteins were quantified using the 2D Quant Kit (GE Healthcare Biosciences, Piscataway, NJ, USA) following the manufacturer's protocol, and either immediately used in isoelectric focusing procedures or stored at −80°C.

For first-dimensional isoelectric focusing, 250 μL sample containing 100 μg proteins were loaded onto the immobilized pH gradient (IPG) strip, pH 4—7 (GE Healthcare Biosciences). Isoelectric focusing was carried out via stepwise voltage increment under the following conditions: step 1, rehydration, 30 V for 12 hours; step 2, 200 V (gradient) for 0.5 hour; step 3, 300 V for 0.5 hour; step 4, 500 V for 1 hour; step 5, 1000 V for 1 hour; step 6, 4000 V for 1 hour; step 7, 6000 V for 1 hour; step 8, 8000 V for 60 kVh; total, 64 kVh. After that, the strips were first equilibrated on an orbital shaker for 15 minutes in equilibration buffer [50mM Tris, pH 8.8; 6M urea; 30% v/v glycerol; 2% w/v sodium dodecyl sulfate (SDS); and trace bromophenol blue] (all were from Sigma Co., (St. Louis, MO, USA) except glycerol, which was from Wako, Osaka, Japan) containing 1% w/v D, L-dithiothreitol, and subsequently in the same buffer with 2.5% w/v iodoacetamide (Sigma) replacing D, L-dithiothreitol for another 15 minutes. The equilibrated strip was then transferred onto the second-dimensional SDS-PAGE gel and sealed with 0.5% agarose. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% polyacrylamide gel (13 cm × 13 cm × 1.5 mm) at a constant current of 10 mA for 1 hour and 40 mA for 3.5 hours. Sypro Ruby stain was used for visualization of the *two-dimensional gel electrophoresis* (2-DGE).

Image analysis, in-gel tryptic digestion, and mass spectrometry analysis

Gel images were captured using Typhoon 9200 Variable Mode Imager (GE Healthcare Biosciences), and digitalized

images were analyzed with the PDQuest version 7.3.1 software (Bio-Rad, Hercules, CA, USA) for spot detection, match, quantification, and comparative analyses. The theoretical molecular mass (Mr) and isoelectric point (pI) values of the 2-DE markers were used to calibrate the Mr and pI of the protein spots in the 2-DGE. Intensity levels were normalized between gels as a proportion of the total protein intensity, and protein quantity of each spot was calculated by integrating the density over the spot area.

The four chosen protein spots were manually excised from the gel and cut into 1–2 mm^3 gel pieces. The gel pieces were reduced with 50mM dithioerythritol in 25mM ammonium bicarbonate, pH 8.5, at 37°C for 1 hour, and subsequently alkylated with 100mM iodoacetamide in 25mM ammonium bicarbonate, pH 8.5, at room temperature in the dark for 1 hour. The pieces were then washed twice with 50% acetonitrile (ACN) in 25mM ammonium bicarbonate, pH 8.5, for 15 minutes each time. After that, the pieces were dehydrated with 100% ACN for 5 minutes, dried, and then rehydrated with 10 μL mix solution of 0.0225 μg sequencing grade modified trypsin (Promega, Madison, WI, USA) in 25mM ammonium bicarbonate, pH 8.5, followed by incubation in 37°C for 16 hours. The tryptic peptides were extracted twice with 50% ACN containing 5% formic acid for 15 minutes each time with moderate sonication (on ice). The extracted solutions were pooled and evaporated to dryness under vacuum.

Protein spots were subjected to concerted matrix-assisted laser desorption–ionization (MALDI) peptide mass fingerprinting and collision-induced dissociation tandem mass spectrometry (CID MS/MS) analysis for protein identification using a dedicated Q-TOF Ultima MALDI instrument (Micromass, Manchester, UK). Samples were premixed with matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN, 0.1% v/v trifluoroacetic acid, and 2% w/v ammonium citrate) in a 1:1 ratio and spotted onto the 96-well MALDI sample stage. MALDI MS were searching peaks within the m/z 800–3000, and the count more than 150 were switching to CID MS/MS using argon as collision gas. Masses of trypsin peptides occurring at m/z 842.509 and 2211.104 were used for internal calibration.

Data search and statistical analysis

After data acquisition, the files were searched by querying the SwissProt and/or NCBI database using MASCOT (http://www.matrixscience.com). All values represented triplicate results, and the mean averages and standard deviations were calculated. The data were then compared by Duncan's multiple range method using SPSS V12 statistical analysis software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Selection of housekeeping genes and evaluation of their gene expressions

All the *E. coli* O157:H7 used in this study have the *stxII* gene and were isolated from clinics and cattle feces in Taiwan. The infective ability of these stains has not yet been verified.[10] At present, there are no epidemic outbreaks involving any locally isolated *E. coli* O157:H7 in Taiwan.[9] Previously, we developed a reverse transcription real-time PCR assay for the identification and quantification of *E. coli* O157:H7.[14] This assay was used in the present study to evaluate the expressions of *stxII* and *eaeA* by TWC01 that were subjected to stresses. For relative quantification analysis, the presence of strain-specific housekeeping genes must be confirmed. These genes are responsible for the maintenance of basic cellular functions and are constantly expressed in cells under various growth conditions. We examined *E. coli* O157 somatic antigen gene (rfb_{O157}) to determine if it is consistently expressed in different growth periods, in order to determine whether rfb_{O157} is a suitable housekeeping gene for relative quantification. The level of rfb_{O157} gene expression was positively correlated with bacterial population, and there was no significant difference in the ratio of rfb_{O157} gene expression to cell numbers during different growth periods ($n = 3$; $p > 0.05$). In addition, rfb_{O157} is characteristic of *E. coli* O157:H7. We therefore selected it as the housekeeping gene for use in relative quantification analysis of *stxII* and *eaeA*.

Growth curve of *E. coli* O157:H7 after chemical and low-temperature treatments

Growth rates of TWC01, TWE01, and TWE04, isolated from a clinical case and from cattle feces in Taiwan, were analyzed. The growth curve in Fig. 1A shows no difference between the growth rates of these three strains. Two hours after inoculation, growth of the cells reached the log phase, and was in the stationary phase at 6 hours after inoculation. Fig. 1B indicates that TWC01 did not show a delay in growth when subjected to five different stress conditions, including temperature, LA, and HCl stresses. However, it exhibited a stress-induced growth delay under FA or NaClO stresses. When stressed by the presence of FA or NaClO, TWC01 only began to grow after 9 hours and 12 hours, respectively. Despite these growth delays, the overall growth of TWC01 was not affected by these two chemical substances, and the growth rates and trends were the same for these treatment groups as those for the other groups.

Effect of *E. coli* O157:H7 isolated from Taiwan on *stxII* and *eaeA* expressions under normal conditions

Fig. 2 shows that there was no significant difference in *stxII* gene expression between the three strains, which were isolated from Taiwan after 2 hours of incubation ($n = 3$; $p > 0.05$). Nevertheless, the *stxII* expression by the TWC01 was 1.5 times and 1.8 times that of the TWE01 and TWE04 after 4 hours and 6 hours of incubation, respectively ($p < 0.05$). However, *stxII* expression in TWC01 was suppressed after 8 hours of incubation. There was no significant difference in *eaeA* expression between the three strains after 2 hours of incubation ($n = 3$; $p > 0.05$). After 4 hours, however, the *eaeA* expression by the TWC01 was 1.04–1.13 times that of TWE01 and TWE04 ($p < 0.05$). The expression of *eaeA* continued to be greater in TWC01 than in TWE01 and TWE04 after 6 hours and 8 hours of incubation. In summary, the results showed that expressions of

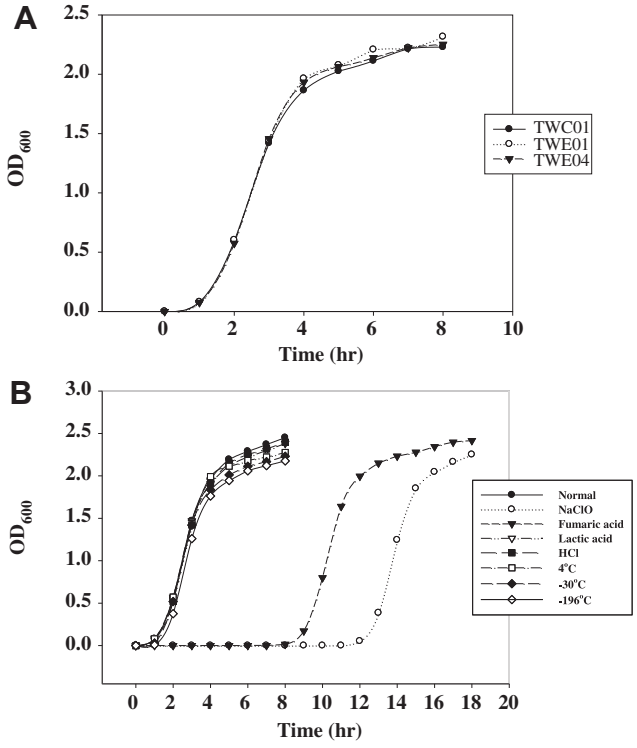

Figure 1. Effect of chemical and low-temperature stresses on the growth curve of *Escherichia coli* O157:H7: (A) Growth of TWC01 (closed circles), TWE01 (open circles), and TWE04 (closed triangles) under normal conditions; and (B) their growth after treatment with NaClO (open circles), fumaric acid (closed triangles), lactic acid (open triangles), or HCl (closed squares) and incubation at 4°C (open squares), −30°C (closed diamonds), or −196°C (open diamonds). HCl = hydrochloric acid; NaClO = sodium hypochlorite; TWC01 = *E. coli* O157:H7 TWC01; TWE01 = *E. coli* O157:H7 TWE01; TWE04 = *E. coli* O157:H7 TWE04.

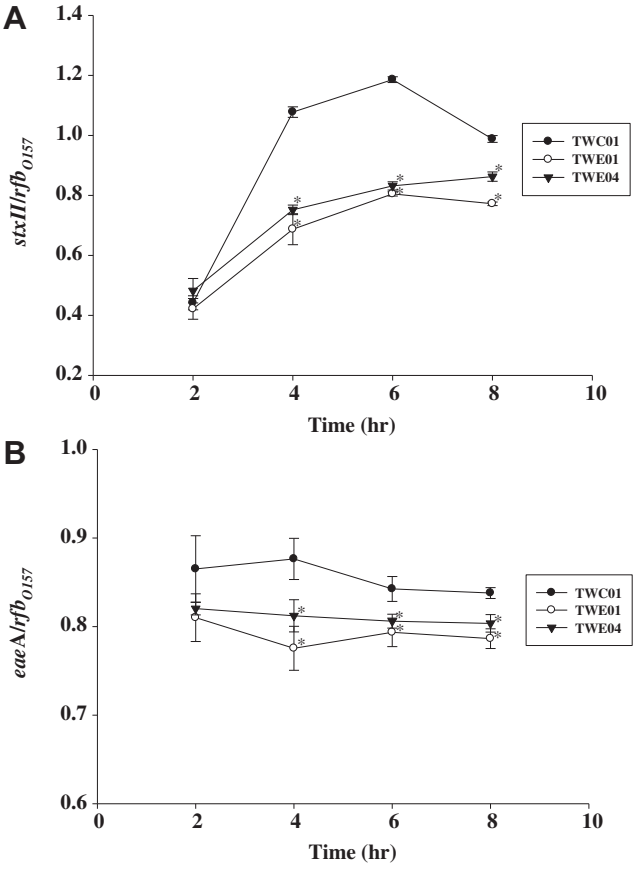

Figure 2. Relative quantification of the gene expressions of (A) *stxII* and (B) *eaeA* in TWC01, TWE01, and TWE04 using reverse transcription real-time PCR and a TaqMan system. A statistical comparison was made using ANOVA, followed by Duncan's test. *$p < 0.05$ versus TWC01. ANOVA = analysis of variance; PCR = polymerase chain reaction; TWC01 = *E. coli* O157:H7 TWC01; TWE01 = *E. coli* O157:H7 TWE01; TWE04 = *E. coli* O157:H7 TWE04.

stxII and *eaeA* in an *E. coli* O157:H7 isolated from a clinical case was higher than those in strains isolated from cattle feces.

Gene expressions of *stxII* and *eaeA* in TWC01 after chemical treatments and adaption

The results of our previous research and in this study showed that the virulent factor gene expression of TWC01 from the clinical case was higher than that from cattle feces. Expressions of *stxII* and *eaeA* in TWC01 were further analyzed under stresses, including food preservation (involving acid and temperature challenges) and detergents used for environmental hygiene (NaClO and FA). Fig. 3A shows the *stxII* expression by TWC01 after four different chemical stress treatments. The expression of *stxII* in the HC1 and FA treatment groups in the initial log phase was higher than that in the untreated and LA treatment groups ($p < 0.05$), whereas the expression in the LA treatment group was significantly lower than that in the control group. In the mid log phase, no significant difference in *stxII* expression was observed between the LA, HCl, and FA treatment groups ($p > 0.05$). However, the *stxII* expression in all four stress groups was higher than that in the control group ($p < 0.05$). Among the treatment groups, the increase in the rate of *stxII* expression from the initial log phase to the mid log phase was highest in the LA treatment group and lowest in the NaClO treatment group. This indicates that LA stress can greatly increase the *stxII* expression of strains during the log phase. Moreover, in the initial stationary phase, the *stxII* expression of each treatment group increased, especially the HCl treatment group, in which expression increased by around 1.2 times the rate of the control group. Finally, when the cells reached the middle stationary phase, gene expressions for all treatment groups, except LA, which continued to increase, began to decrease. However, the expression rate of *stxII* remained significantly higher in the treatment groups than in the control group ($p < 0.05$).

Fig. 3B shows the *eaeA* expression of TWC01 under four different chemical stress treatments. In the initial and mid log phases, the *eaeA* expression of the LA and NaClO treatment groups was significantly higher than that of the control and other treatment groups ($p < 0.05$). This was especially true for the LA treatment group, in which the

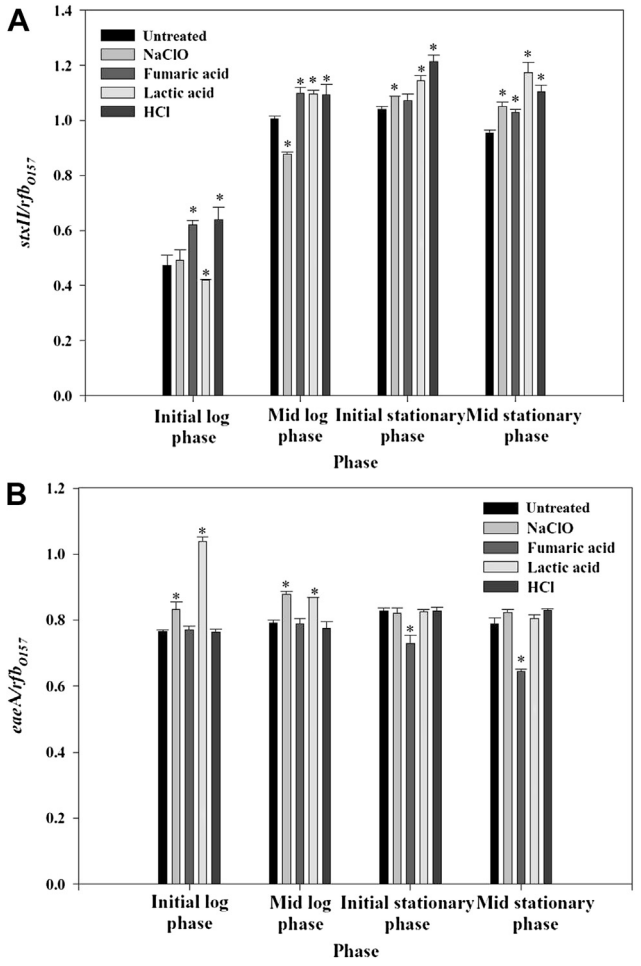

Figure 3. Expressions of (A) *stxII* and (B) *eaeA* in TWC01 after treatment with NaClO, fumaric acid, lactic acid, or HCl. A statistical comparison was made using ANOVA, followed by Duncan's test. *$p < 0.05$ versus untreated TWC01. ANOVA = analysis of variance; HCl = hydrochloric acid; NaClO = sodium hypochlorite; TWC01 = *E. coli* O157:H7 TWC01.

eaeA expression rate was 1.45 times that of controls. No significant difference in *eaeA* expression was observed among the groups, except in the FA group when the cells reached the stationary phase ($p > 0.05$). Previous studies have indicated that *eaeA* gene was influenced by growth phase and growth pH. Expression levels increased during exponential-phase growth at pH 5.5, but no pH-related differences were observed in cells in the stationary phase. This result was due to the interaction of the acid response system with other specific and newly expressed *eaeA* regulators.[15] The same phenomenon has been observed in this study. As the results of chemical adaption of TWC01, there was the same trend of virulent factor gene expressions, comparing with chemical stress treatment (data not shown). It also accorded with our previous results, indicating that the protein expression of shiga-like toxin was increased.[7] In summary, the *stxII* expression of chemically treated and adapted TWC01 cells significantly increased when cells entered the stationary phase, and cells in the HCl treatment group exhibited the greatest increase in expression. The expression of *eaeA* increased in the initial log phase after LA treatment and adaption, suggesting that both organic and inorganic acids enhance the expressions of virulence genes in these strains.

Gene expressions of *stxII* and *eaeA* in TWC01 after low-temperature treatments

TWC01 was treated at three different low temperatures. The resultant *stxII* and *eaeA* expressions were analyzed, which are shown in Fig. 4A and B, respectively. In TWC01 treated at −30°C, the *stxII* expression 2 hours and 4 hours after inoculation was significantly lower than that in the control group and groups treated at 4°C and −196°C ($p < 0.05$). When treated at 4°C, the *stxII* expression at 6 hours and 8 hours after inoculation was significantly lower than that for controls ($p < 0.05$).

In TWC01 treated at −196°C, the *eaeA* expression was significantly lower at 2 hours and 4 hours after inoculation than that for the control group and groups treated at 4°C and −30°C ($p < 0.05$), but the *eaeA* expression showed an

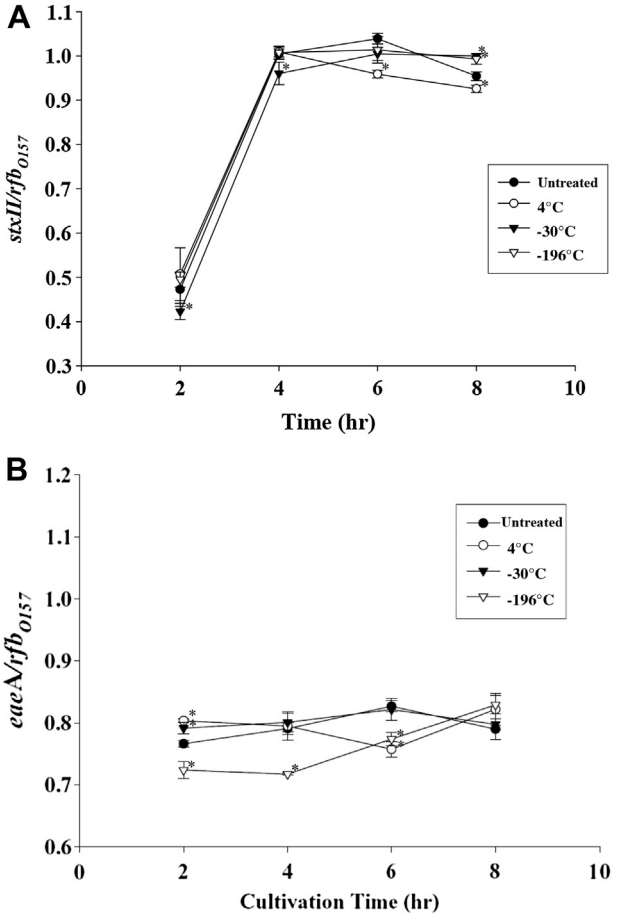

Figure 4. Expression of (A) *stxII* and (B) *eaeA* gene expressions in the TWC01after incubation at 4°C, −30°C, or 196°C. A statistical comparison was made using ANOVA, followed by Duncan's test. *$p < 0.05$ versus untreated TWC01. ANOVA = analysis of variance; TWC01 = *E. coli* O157:H7 TWC01.

increasing trend when cells reached the stationary phase (6 hours and 8 hours after inoculation). No significant difference was observed in the *eaeA* expression at 8 hours after inoculation between the control group and groups treated at 4°C, −30°C, and −196°C ($p > 0.05$). The *eaeA* expression of the 4°C stress group was significantly higher than that of the control and −196°C groups ($p < 0.05$) 2 hours after inoculation. No significant difference was observed in the expression of *eaeA* between the 4°C stress and control groups 4 hours after inoculation ($p > 0.05$). Six hours after inoculation, the *eaeA* expression was significantly lower in cells treated at 4°C and −196°C than in cells of the control group and those treated at −30°C ($p < 0.05$). Moreover, there was no significant difference in the *eaeA* gene expression of temperature-stress groups and the control group 8 hours after inoculation ($p > 0.05$). In summary, *stxII* and *eaeA* expressions were downregulated in the stationary phase at 4°C, whereas the *eaeA* expression was downregulated 2 hours after inoculation at −196°C. It has been reported that low temperatures (4°C) can reduce acid tolerance of strains, suggesting that temperature stress has effects on the biological functions and activities of strains.[15,16] Our findings indicate that low-temperature storage does not induce an increase in *stxII* and *eaeA* expressions, and is therefore associated with a lower expression of virulence genes in *E. coli* O157:H7 than are chemical stresses.

Effect of acid adaption on the outer membrane of TWC01 as determined by 2-DE

The results of our previous research and this study showed that HCl and LA exhibited more influence on these virulent factors and total protein expression. In this study, these two acids were used to investigate the effect on the outer membrane proteins of acid-adapted TWC01. Various exposed proteins on the surface of pathogens interact with the host directly through adherence or interaction with different receptors. Once adhered, the pathogen can begin colonization of the gastrointestinal tract.[17,18] The periplasmic and outer membrane proteins of *E. coli* are required for signal transduction.[19–21] Outer membrane proteins extracted from untreated TWC01 and TWC01 treated with HCl or LA were subjected to 2-DE (Fig. 5). The gel profile was then analyzed using ImageMaster 2D Platinum 5.0 software (GE Healthcare Life Sciences, Piscataway, NJ, USA) that selected proteins with a onefold or greater increase/decrease of protein spots (or no expression). Four of these selected protein spots were subjected to in-gel digestion prior to analysis using a NanoLC-MS system. The relative expression of each selected protein and the results of a Mascot database search are shown in Table 1.

Spot 7 protein was identified as an outer membrane porin protein C (OmpC), a hydrophilic small molecule and nonspecific channel protein from Gram-negative bacteria. The expression of OmpC decreased 3.6-fold after HCl treatment and was not detected after LA treatment. OmpC is composed of 16 β-strands, formed into a β-barrel domain, allowing ions and hydrophilic molecules to be imported and exported through the cell membrane. Spot 12 protein was identified as an outer membrane protein A (OmpA), a channel protein of *E. coli*. The expression of OmpA increased 2.38-fold after HCl treatment and decreased 1.9-fold after LA treatment. OmpA is a major outer membrane protein of *E. coli*, and its main functions include nonspecific small molecule diffusion, maintaining structural integrity of the cell surface, and interacting with F-factor for bacterial conjugation. Additionally, Torres and Kaper[22] (2003) reported that OmpA is essential for interaction with hosts and for adherence of enterohemorrhagic *E. coli* to intestinal epithelial cells. Moreover, Shin et al[23] reported that OmpA is responsible for adherence to human brain microvascular endothelial cells, resulting in *E. coli* invasion through the β-barrel domain protruding from the cell surface. Thus, upregulation of OmpA expression is important for the adherence of the pathogen to host cells. Spot 15 protein was identified as an outer membrane protein X (OmpX). The expression of OmpX increased 1.61-fold after HCl treatment and decreased 3.9-fold after LA treatment. OmpX is required for the adherence of the pathogen to mammalian cells and survival inside macrophages.[24,25] Spot 8 protein was identified as a chain A, elongation factor complex Ef-TuEF-Ts from *E. coli*. The expression of this protein increased 2.9-fold after HCl treatment and 1.38-fold after LA treatment. Elongation factor Tu, a guanine nucleotide-binding protein, is implicated in protein synthesis. For translation of mRNA, elongation factor Tu is activated by binding to guanosine triphosphate (GTP) and transporting aminoacyl-tRNA (aa-tRNA) to the A site on the ribosome.[26,27]

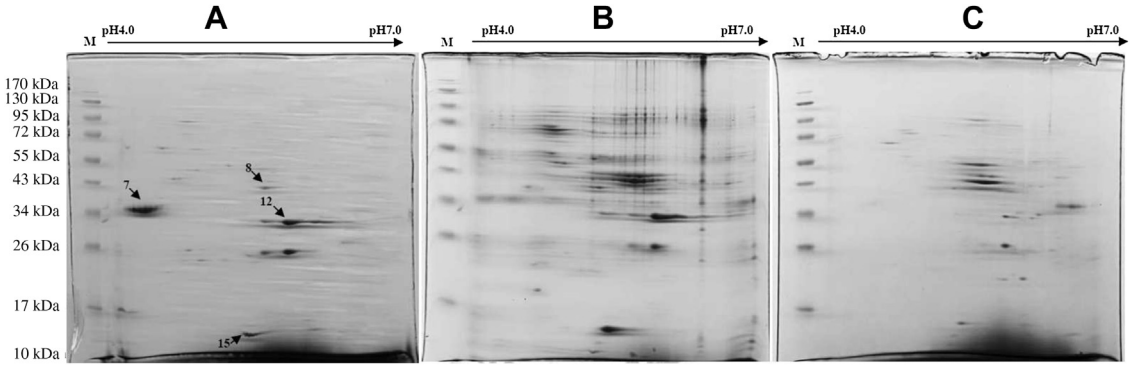

Figure 5. Analysis of outer membrane protein expression of acid-adapted *E. coli* O157:H7 using two-dimensional electrophoresis: (A) untreated, (B) treated with HCl, and (C) treated with lactic acid. HCl = hydrochloric acid.

Table 1 Characteristics and relative protein expressions of four spots from acid-adapted *Escherichia coli* O157:H7 separated using two-dimensional electrophoresis

Spot	Protein description	Mascot result			Mr	pI	Relative protein expression	
		Accession no.	Score	SC (%)			HCl	LA
7	Outer membrane porin protein C	NP_288795	84	5	40,483	4.55	0.27×	No expression
8	Chain A, elongation factor complex Ef-TuEF-Ts from *E. coli*	1EFU_A	228	11	42,150	5.22	2.90×	1.38x
12	Outer membrane protein A	NP_286832	491	26	37,178	5.99	2.38	0.52×
15	Chain A, crystal structure of the outer membrane protein OmpX from *E. coli*	1QJ8_A	177	43	16,350	5.04	1.61	0.26×

HCl = hydrochloric acid; LA = lactic acid; Mr = molecular mass; OmpX = outer membrane protein X; SC = percentage sequence coverage.

Strong acids, like HCl, are completely dissociated in water (or a medium), leading to the rapid development of a proton gradient between inner and outer bacteria. Organic acids, like LA, diffuse and then dissociate inside bacteria cells during acid treatments, and result in a miner proton gradient, compared with strong acids. Previous studies have shown that OmpC and OmpF are required for membrane permeability. *E. coli* O157:H7 is able to survive in the human intestine along with a variety of intestinal microflora and can colonize host intestines, even in a low pH environment that contains many antibacterial substances (such as bile salts). Numerous different stresses during *E. coli* growth, survival, and infection lead to *E. coli* responses that increase survival rate. These responses improve resistance to membrane permeability, limit the entry of substances such as acids and salts into the cytoplasm, and reduce osmotic gradients between intracellular and extracellular fluids.[28,29] Sainz et al[30] reported that acid stress resulted in a reduction of outer membrane protein expression in pathogenic *E. coli* isolated from fermented foods and *E. coli* O157: H7. Additionally, Sato et al[31] observed that the expressions of OmpC and OmpF are regulated by osmolarity and acid pH. We also found that the expression of OmpC is repressed under organic acid adaption and suggest that downregulation of OmpC in *E. coli* O157: H7 in the presence of both acids leads to a change in membrane permeability. Expressions of four outer membrane proteins of TWC01 changed after HCl or LA treatment. HCl treatment reduced the expressions of channel proteins, resulting in a decrease in ion permeability of the outer membrane. This limits the number of ions, H^+, and hydrophilic molecules that pass through the outer membrane, resulting in increased acid tolerance.

Sugawara and Nikaido[32] indicated that ompA functions as the major channel for proton influx across the outer membrane. Moreover, previous studies have indicated that the functional channel gating mechanism was also shown to contribute to the survival of bacteria under osmotic stresses.[33] In the current study, HCl adaption enhanced the expressions of ompA and ompX proteins, which also have the adherence-related functions to reduce the number of protons and protect bacteria cells, resulting in concentration of ions inside the cells. This suggests that TWC01 responds to HCl treatment through induction of acid tolerance and enhancement of adhesion. The undissociated weak acid LA interacts with phospholipids, lipopolysaccharides, and diffusion channel proteins on the outer membrane and represses its stability, causing inhibitory effect on the bacteria.[34] Our results indicate that nonspecific diffusion channel proteins ompA and ompX were repressed in LA-adapted *E. coli* O157:H7, reducing the bacteria cells contacted with LA and adaption the reversed conditions. The reduced expression of these proteins after LA treatment results in a reduced ability of TWC01 to adhere to host cells. In conclusion, the results showed that the gene expressions of *stxII* and *eaeA* in *E. coli* O157:H7 isolated from a clinical case were higher than that from cattle feces. Both acids and NaClO enhance the expressions of these virulent factor genes in TWC01. In addition, the outer membrane proteins of TWC01 were regulated under HCl adaption, indicating induction of acid tolerance and enhancement of adhesion in TWC01. LA adaption of TWC01 resulted in downregulation of channel protein expression and reduced expression of adherence-related proteins. The results of this study are helpful for understanding the acid resistance properties of locally isolated *E. coli* O157:H7 and improving the ability of health officials to control this pathogen in Taiwan.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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