Journal of Food Safety

Journal of Food Safety ISSN 1745-4565

NACL INFLUENCES THERMAL RESISTANCE AND CELL MORPHOLOGY OF *ESCHERICHIA COLI* STRAINS

HEEYOUNG LEE[1], SOOMIN LEE[1], SEJEONG KIM[1], JIMYEONG HA[1], JEEYEON LEE[1], KYOUNG-HEE CHOI[2] and YOHAN YOON[1,3]

[1]Department of Food and Nutrition, Sookmyung Women's University, Seoul 140-742, Korea
[2]Department of Oral Microbiology, College of Dentistry, Wonkwang University, Jeonbuk, Korea

[3]Corresponding author.
TEL: 82-2-2077-7585;
FAX: 82-2-710-9479;
EMAIL: yyoon@sookmyung.ac.kr

Received for Publication February 17, 2015
Accepted for Publication June 15, 2015

doi: 10.1111/jfs.12213

ABSTRACT

This study elucidated the effect of NaCl on the thermal resistance and cell morphology of *Escherichia coli* strains. *E. coli* O157:H7 NCCP11142, *E. coli* O111 ATCC12795 and *E. coli* O26 ATCC43887 were incubated in tryptic soy broth formulated with 0% NaCl (nonhabituated) as well as broth formulated with 2% and 4% NaCl (NaCl-habituated) at 35C. Nonhabituated, 2% NaCl-habituated and 4% NaCl-habituated *E. coli* O157:H7, *E. coli* O111 and *E. coli* O26 were heat-challenged at 50C. The transcriptomes (osmotic stress-related: *betA*, *ompC*, *proV*, *proW* and heat stress-related: *clpB*, *dksA*, *dnaJ*, *dnaK*, *grpE*, *msbB*, *rpoE*) were analyzed using real-time polymerase chain reaction and the morphology of *E. coli* strains was also observed by field emission scanning electron microscopy. The survival of *E. coli* O157:H7 was higher ($P < 0.05$) than those of other strains after the heat challenge. The expression levels of *proV*, *proW* and *rpoE* increased ($P < 0.05$) as NaCl concentration increased, but only *proV* expression levels were significantly different ($P < 0.05$) among *E. coli* strains. The results indicate that the effect of NaCl on the thermal resistance and the cell morphology depends on the *E. coli* strain, and increased *proV* expression level by NaCl may cause the increased thermal resistance of *E. coli* O157:H7.

PRACTICAL APPLICATIONS

NaCl is widely used as an antimicrobial and a preservative agent in food processing. However, *Escherichia coli* O157:H7 exposed to 2% and 4% NaCl showed thermal resistance, upregulated gene expression on osmotic- and heat-related genes and changed cell morphology. Thus, NaCl concentration should be limited in food not to increase *E. coli* O157:H7 tolerance to other stresses.

INTRODUCTION

The pathogenic *Escherichia coli* strains associated with gastrointestinal infections are categorized into enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, diffuse-adherent *E. coli* and Shiga toxin-producing *E. coli* (STEC) (Bettelheim 2007). In particular, STEC, which causes hemorrhagic colitis and the hemolytic uremic syndrome (HUS) in human, produces Shiga toxins, induces attaching and effacing lesions and includes pO157 (60-Mda plasmid) (Nataro and Kaper 1998).

In the U.S.A., the most common serotype in STEC gastrointestinal tract infections is O157, and the serotype is estimated to be responsible for 73,000 illnesses annually with 2% to 15% of HUS development (Mead *et al.* 1999; Dundas *et al.* 2001; Rangel *et al.* 2005; Tserenpuntsag *et al.* 2005). Moreover, a survey by Mead *et al.* (1999) showed that non-O157 STEC causes an estimated 37,000 illnesses annually in the U.S.A. The most common non-O157 STEC between in 1983 and in 2002 was *E. coli* O26, followed by *E. coli* O111 (Brooks *et al.* 2005). From 2007 to 2009 in European Union/European Economic Area countries, *E. coli* O26 was the second highest serogroup associated with HUS

cases after *E. coli* O157 (European Centre for Disease Prevention and Control/European Food Safety Authority 2011). In 2008, an *E. coli* O111 outbreak occurred in Oklahoma, causing 341 illnesses, and HUS was identified in 26 (16.7%) of 156 confirmed or probable *E. coli* O111 infections; 65.4% of patients with HUS had dialysis and one patient died (Piercefield *et al.* 2010). Thus, non-O157 STEC strains have also become significant in food safety as much as *E. coli* O157.

NaCl is added to food as an antimicrobial and a preservative, however, foodborne pathogens have been isolated even from fish and meat products treated with high concentration of NaCl (Hajmeer *et al.* 2006; Cabedo *et al.* 2008). In food, NaCl may change physiological characteristics of bacteria such as biofilm formation, increased lag phase duration, stress responses, as well as cell morphology (Oscar 1999; Garner *et al.* 2006; Choi and Yoon 2013; Yoon *et al.* 2013). The changes in physiological characteristics may thus affect bacterial responses to various environments, which may influence food safety.

Therefore, the objective of this study was to elucidate the effect of NaCl on the thermal resistance and cell morphology of various *E. coli* strains. In addition, the transcriptional analysis was also conducted to identify the genes related to the responses of *E. coli* strains to NaCl.

MATERIALS AND METHODS

Inocula Preparation

E. coli O157:H7 NCCP11142, *E. coli* O111 ATCC12795 and *E. coli* O26 ATCC43887 were cultured in 10 mL tryptic soy broth (TSB) (Difco, Becton Dickinson and Company, Sparks, MD) at 35C for 24 h. The 0.1 mL portions of the culture were transferred into 10 mL fresh TSB for subculture at 35C for 24 h. The subcultures were then centrifuged at $1,912 \times g$ and 4C for 15 min. The cultures were washed twice with phosphate buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4, 8.0 g of NaCl and 0.2 g of KCl in 1 L of dH_2O), followed by serial dilution with PBS to obtain 4 log cfu/mL.

NaCl Habituation

The 0.1 mL portions of the inocula were inoculated into 10 mL TSB supplemented with 0% NaCl for nonhabituated cells and 2% NaCl and 4% NaCl for NaCl-habituated cells, followed by incubation at 35C until *E. coli* strains reached the early stationary phase at all NaCl concentration. These NaCl-habituated and nonhabituated strains were then harvested by centrifuging at $1,912 \times g$ under 4C for 15 min and washed twice with PBS, and these NaCl-habituated and non-habituated *E. coli* strains were used for further experiment.

Thermal Resistance

The 0.1 mL portions of nonhabituated, 2% NaCl-habituated and 4% NaCl-habituated *E. coli* O157:H7, *E. coli* O111 and *E. coli* O26 were inoculated to 10 mL TSB, TSB+2% NaCl and TSB+4% NaCl, respectively, and they were preheated at 50C in a water bath. The survivals of the *E. coli* strains were then enumerated on tryptic soy agar (Difco) at 0, 30, 60, 90 and 120 min at 50C, and the plates were incubated at 35C for 24 h. Bacterial cell counts (cfu/mL) were expressed as log (Y_t/Y_0), Y_t is the number of *E. coli* cell counts at time t, and Y_0 is the number of initial bacterial counts.

Transcriptional Analysis

Transcriptional analysis was conducted to evaluate the effect of NaCl on the expression of heat and osmotic stress-related genes. For transcription analysis, the genes of *E. coli* were searched in PubMed (assession No. AE005174) and primers were designed with sequences. The 0.4 mL portions of each *E. coli* O157:H7, *E. coli* O111 and *E. coli* O26 inocula were inoculated in 40 mL TSB, followed by incubation at 35C by $OD_{600} = 0.5$. To expose *E. coli* strains to 0, 2 and 4% NaCl, 9 mL of each *E. coli* culture was mixed with TSB for 0% NaCl and TSB was supplemented with 30% NaCl at appropriate ratio for 2 and 4% NaCl for 20 min. The 1.5 mL proportions of each culture were centrifuged at $5,000 \times g$ at room temperature for 5 min, and the bacterial cell pellets were resuspended in 200 μL of lysozyme (mg/mL) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The suspensions were incubated at 35C for 15 min and vigorously vortexed every 5 min. For RNA extraction, RNeasy Mini Kit (Qiagen, Hilden, Germany) was used and analysis was conducted according to the manufacturer's guideline. After RNA extraction, the concentrations of total RNA were measured with Epoch Micro-Volume Spectrophotometer System (Bio Tek Instruments, Winooski, VT). The total RNA of *E. coli* O157:H7, *E. coli* O111 and *E. coli* O26 exposed to 0, 2 and 4% NaCl were reverse-transcribed into cDNA. Reverse transcription was conducted using the QuantiTect Reverse Transcription Kit (Qiagen) in accordance with the manufacturer's protocol. Osmotic (*betA*, *ompC*, *proV* and *proW*) and heat stress-related (*clpB*, *dksA*, *dnaJ*, *dnaK*, *grpE*, *msbB* and *rpoE*) oligonucleotide primers used for quantitative real-time polymerase chain reaction (PCR) are shown in Table 1. The 16S rRNA primers were used as a reference gene. The Rotor-Gene SYBR Green PCR Kit (Qiagen) was used as the PCR reaction mixture [24 μL;

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED IN THE QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

	Gene	Primer	Sequence (5′→3′)	Function	Assession number	Reference
Osmotic stress-related gene	16s rRNA	16s rRNA-F 16s rRNA-R	GAA TGC CAC GGT GAA TAC GTT ACC CAC TCC CAT GGT GTG A	House keeping	–	Parry-Hanson *et al*. (2010)
	betA	betA-F betA-R	CAT CTC GTC GTA GCC CAT TT GCT CGA TGA GTT TGT GCG TA	Enzyme-related osmotic adaptation	AE005174	This study
	ompC	ompC-F ompC-R	TGC CCT GGA TCT GAT ATT CC CTA CAT GCG TCT TGG CTT CA	Produce outer membrane protein	AE005174	This study
	proV	proV-F proV-R	GCG GGC AAG TAC TGA TTG AT ACT GGA AGA CCA TCG CAA TC	Transport of small molecules	AE005174	This study
	proW	proW-F proW-R	ACT TCG TGG TAT CGG TCG TC GAG GAT AAT GGC GAG GAT CA	Transport of small molecules	AE005174	This study
Heat stress-related genes	*clpB*	clpB-F clpB-R	CCT TCA ACG ATG GCA GTT TT AAA TTC GCC GTA CCA TTC AG	Macromolecule degradation	AE005174	This study
	dksA	dksA-F dksA	GTT GAT TAC GCC ATG CTT CC CCG GGC GAA GAG TAT ATG AA	Micromolecule synthesis	AE005174	This study
	dnaJ	dnaJ-F dnaJ-R	GCG GTG CTG ATT TAC GCT AT CTC TTC CAG AGT CGG AAT GC	Produce heat shock protein	AE005174	This study
	dnaK	dnaK-F dnaK-R	TCG TAT GCC AAT GGT TCA GA AGC ACC GAT TGC TAC AGC TT	Folding and ushering protein	AE005174	This study
	grpE	grpE-F grpE-R	GCA CGA TCC AGG CTA TCA AT CGT CGT CGT ACT GAA CTG GA	Produce heat shock protein	AE005174	This study
	msbB	msbB-F msbB-R	AGC ATG GCA GGA ATA TCG AC AGA TGC GGC GTA ATA ACG AG	Adaptation of atypical condition	AE005174	This study
	rpoE	rpoE-F rpoE-R	GCC TGA TAA GCG GTT GAA CT AGC CGC TAT CAT GGA TTG TC	Regulator, produce heat shock stress	AE005174	This study

6.5 μL of dH_2O, 12.5 μL of master mix and 2.5 μL of 10 pmol/μL primers (forward and reverse)]. PCR strips were filled with the PCR reaction mixture (24 μL) and 1 μL of cDNA. To compare the expression levels of heat shock genes, Rotor-Gene Q software (Qiagen) was used and the threshold cycle (C_T) values of the genes were adjusted to 0.1.

Field Emission Scanning Electron Microscopy

To examine the morphology of *E. coli* O157:H7, *E. coli* O111 and *E. coli* O26 exposed to 0, 2 and 4% of NaCl, the *E. coli* strains were cultured with sterilized cover glass (0.5×0.5 cm^2) in TSB (35C, 24 h). The coverglasses were subjected to TSB supplemented with 0, 2 and 4% NaCl for 1 h in a sterile 24-well flat-bottomed polystyrene microtiter plate. The coverglasses were aseptically transferred to other sterile wells and 30 μL of 1.8% glutaraldehyde (Sigma-Aldrich Co., St. Louis, MO) was carefully added above the coverglasses for fixation for 30 min at room temperature. The coverglasses were then washed three times with 1 mL of dH_2O at room temperature for 5 min at each step, followed by a second fixation with 2% osmium tetroxide (Sigma-Aldrich Co.) for 20 min. Next, they were washed three times with 1 mL of dH_2O at room temperature for 5 min. The fixed bacterial cells were gradually dehydrated with 25, 50, 75, 90 and 100% ethyl alcohol and hexamethyldisilazane (Sigma-Aldrich Co.). The samples were eventually coated using a sputter coater (108 auto; Cressington Scientific Instruments Ltd., Watford, England) and the bacteria cells were observed using field emission scanning electron microscopy (JSM-7600F, JEOL Ltd., Tokyo, Japan).

Statistical Analysis

All the experiments were repeated twice with two samples per repeat ($n = 4$). Data for thermal resistance and gene expression of osmotic and heat stress-related genes were analyzed using the general linear model procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). The mean differences among interactions were separated with a pairwise *t*-test at the significance level of alpha = 0.05.

RESULTS AND DISCUSSION

All *E. coli* strains gradually decreased during the heat challenge at 50C for 120 min, regardless of exposed NaCl concentrations (Table 2). *E. coli* O157:H7 cells habituated to 4% NaCl had significantly lower ($P < 0.05$) reduction than the nonhabituated and 2% NaCl-habituated *E. coli* O157:H7. However, nonhabituated *E. coli* O111 and *E. coli* O26 had generally lower ($P < 0.05$) cell reductions compared with those of habituated *E. coli* O111 and *E. coli* O26 to 2% and 4% NaCl (Table 2). In a study by Blackburn *et al.* (1997), the *D*-value of *E. coli* O157:H7 strains (E30138, E30228,

TABLE 2. REDUCTIONS [MEAN ± STANDARD DEVIATION; LOG (Y_T/Y_0)] IN THE CELL COUNTS OF *ESCHERICHIA COLI* O157:H7, *E. COLI* O111 AND *E. COLI* O26, WHICH WERE NONHABITUATED TO 0% AND NACL-HABITUATED TO 2% AND 4%, DURING HEAT CHALLENGE AT 50C FOR 120 MIN

E. coli strains	Heating time (min)	NaCl (%) 0	2	4
E. coli O157:H7	0	$0.0 \pm 0.0^{A,a}$	$0.0 \pm 0.0^{A,a}$	$0.0 \pm 0.0^{A,a}$
	30	$-1.3 \pm 0.2^{A,b}$	$-2.2 \pm 0.7^{B,b}$	$-0.7 \pm 0.4^{A,b}$
	60	$-2.4 \pm 0.3^{B,c}$	$-3.3 \pm 0.7^{C,c}$	$-1.4 \pm 0.5^{A,b,c}$
	90	$-2.8 \pm 1.0^{B,c}$	$-3.7 \pm 0.8^{C,c,d}$	$-1.6 \pm 0.5^{A,c}$
	120	$-4.0 \pm 0.8^{B,d}$	$-4.4 \pm 0.7^{B,d}$	$-1.7 \pm 0.3^{A,c}$
E. coli O111	0	$0.0 \pm 0.0^{A,a}$	$0.0 \pm 0.0^{A,a}$	$0.0 \pm 0.0^{A,a}$
	30	$-0.9 \pm 0.2^{A,b}$	$-2.4 \pm 0.6^{B,b}$	$-2.3 \pm 0.6^{B,b}$
	60	$-2.0 \pm 0.6^{A,c}$	$-3.8 \pm 0.4^{B,c}$	$-3.2 \pm 0.6^{B,c}$
	90	$-2.5 \pm 1.0^{A,c}$	$-4.1 \pm 0.2^{B,c}$	$-3.7 \pm 0.9^{B,c,d}$
	120	$-3.4 \pm 1.2^{A,d}$	$-5.3 \pm 1.2^{B,d}$	$-4.2 \pm 0.5^{A,d}$
E. coli O26	0	$0.0 \pm 0.0^{A,a}$	$0.0 \pm 0.0^{A,a}$	$0.0 \pm 0.0^{A,a}$
	30	$-1.2 \pm 0.2^{A,b}$	$-2.4 \pm 0.2^{B,b}$	$-2.0 \pm 0.4^{B,b}$
	60	$-1.8 \pm 0.3^{A,b,c}$	$-3.0 \pm 0.2^{B,b,c}$	$-2.7 \pm 0.7^{B,b,c}$
	90	$-2.2 \pm 0.1^{A,c,d}$	$-3.2 \pm 0.5^{B,c}$	$-3.2 \pm 0.8^{B,c,d}$
	120	$-2.8 \pm 0.1^{A,d}$	$-3.4 \pm 0.6^{A,c}$	$-3.6 \pm 0.8^{A,d}$

Different superscript uppercase letters in the same row mean significantly different at $P < 0.05$. Different superscript lowercase letters in the same column mean significantly different at $P < 0.05$.

E30480 and E32511) in TSB at 62.5C increased concurrently with NaCl concentration under simultaneous stress with NaCl and heat. Skandamis *et al.* (2008) exposed a 10-strain mixture of *Listeria monocytogenes* to 10% NaCl and the *L. monocytogenes* cells were sequentially subjected to 57C. The result from the study showed that *L. monocytogenes* cells were heat-sensitized by NaCl compared with the control. When *L. monocytogenes* ScottA was subjected to 0.09, 0.5, 1.0 or 1.5 M NaCl and then inoculated into test media formulated with 0.09, 0.5, 1.0 or 1.5 M NaCl at 60C, *L. monocytogenes* ScottA showed increased thermal resistance (Jørgensen *et al.* 1995). Blackburn *et al.* (1997) simultaneously exposed *Salmonella* Enteritidis P167807 to 0.5% or 8.5% NaCl and to 54.5–64.5C, and observed the cross-protection effect of the pathogen. Gunasekera *et al.* (2008) researched the cross-regulation of osmotic and heat stress responses of *E. coli* K-12 strain under 0.3 M NaCl, and they also found that the cross-regulation increased oxidative-stress regulations (SoxRS and OxyR). However, in our study, only one strain (*E. coli* O157:H7 NCCP11142) of *E. coli* increased thermal resistance from NaCl at 4%, and in the research of Oulkheir *et al.* (2007), thermal resistance also depended on *E. coli* strain. Thus, it can be suggested that the effect of NaCl on increasing the thermal resistance of *E. coli* is strain-dependent, as suggested by Lee *et al.* (2012) and Lianou *et al.* (2006).

To identify the genes related to thermal resistance, quantitative real-time PCR was performed for transcriptional analysis. Of the four osmotic stress-related genes of *E. coli* strains, the expression levels of *betA* and *ompC* genes were not influenced by NaCl concentration. However, the expression levels of other genes (*proV* and *proW*) were increased ($P < 0.05$) as NaCl concentration increased (Table 3). The *proV* was generally expressed ($P < 0.05$) more than *proW*,

TABLE 3. RELATIVE EXPRESSION LEVELS OF OSMOTIC STRESS-RELATED GENES OF *ESCHERICHIA COLI* STRAINS HABITUATED IN NACL (2 AND 4%) AND NONHABITUATED IN NACL (0%)

E. coli strains	NaCl	Osmotic stress-related genes (mean ± standard error) *betA*	*ompC*	*proV*	*proW*
E. coli O157:H7	0%	1.00 ± 0.0^{a}	1.00 ± 0.0^{a}	1.00 ± 0.0^{c}	1.00 ± 0.0^{c}
	2%	5.32 ± 1.1^{a}	0.62 ± 0.1^{a}	32.08 ± 2.4^{c}	45.22 ± 6.4^{c}
	4%	32.15 ± 4.4^{a}	1.07 ± 0.2^{a}	946.18 ± 260.4^{a}	544.86 ± 84.5^{a}
E. coli O111	0%	1.00 ± 0.0^{a}	1.00 ± 0.0^{a}	1.00 ± 0.0^{c}	1.00 ± 0.0^{c}
	2%	3.60 ± 1.1^{a}	0.64 ± 0.0^{a}	9.69 ± 2.0^{c}	15.93 ± 3.5^{c}
	4%	7.90 ± 0.4^{a}	0.49 ± 0.0^{a}	106.00 ± 30.5^{c}	65.73 ± 19.1^{c}
E. coli O26	0%	1.00 ± 0.0^{a}	1.00 ± 0.0^{a}	1.00 ± 0.0^{c}	1.00 ± 0.0^{c}
	2%	8.15 ± 0.3^{a}	0.75 ± 0.1^{a}	160.53 ± 49.2^{c}	$177.62 \pm 46.1^{b,c}$
	4%	13.37 ± 0.8^{a}	0.90 ± 0.1^{a}	624.34 ± 16.4^{b}	$356.03 \pm 0.4^{a,b}$

Different superscript lowercase letters in the same column mean significantly different at $P < 0.05$.

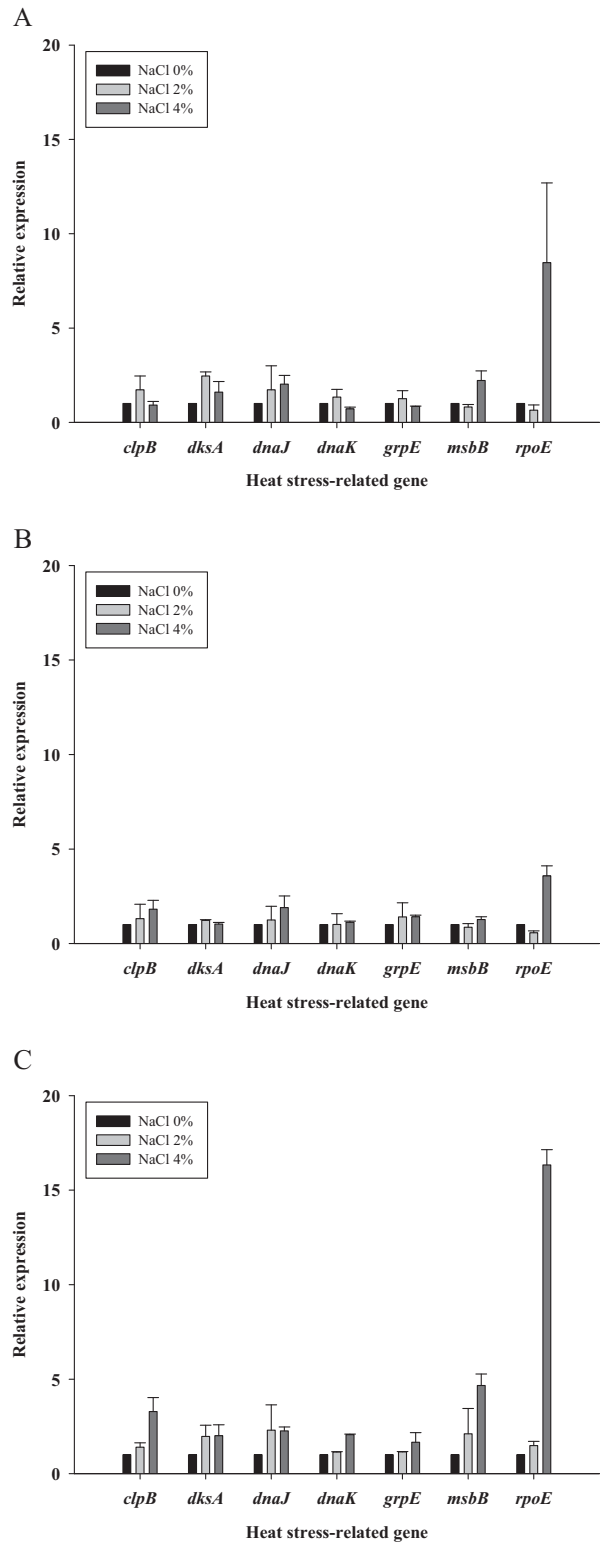

FIG. 1. RELATIVE EXPRESSION LEVELS OF HEAT STRESS-RELATED GENES OF *ESCHERICHIA COLI* STRAINS, HABITUATED IN NACL AND NONHABITUATED IN NACL; (A) *E. COLI* O157:H7, (B) *E. COLI* O111 AND (C) *E. COLI* O26

and *proW* expression was higher ($P < 0.05$) than *betA* and *ompC*. In addition, expression of *proV* in *E. coli* O111 and *E. coli* O26 was lower ($P < 0.05$) at 4% NaCl than in *E. coli* O157:H7, which also showed higher heat resistance than the other strains (Table 2). However, other genes showed no obvious differences in gene expression levels among the *E. coli* strains (Table 3). This result indicates that *proV*, which plays a role in glycine betaine transport, may be related to increase in the thermal resistance of *E. coli* O157:H7 by NaCl. Teixidó *et al.* (2005) showed that accumulation of glycine betaine increased the heat resistance of *Pantoea agglomerans* CPA-2. Thus, it may be that the high expression levels of *proV* in *E. coli* O157:H7 caused glycine betaine accumulation, which resulted in the increased thermal resistance of *E. coli* O157:H7.

The expression levels of heat stress-related genes were measured after their exposure to NaCl to determine whether the expression levels of heat stress-related genes were increased by NaCl, influencing the heat resistance of *E. coli* strains. Of the seven heat stress-related genes, only the expression level of *rpoE*, which responds to temperature changes or oxidative stress (Rouvière *et al.* 1995; De Las Peñas *et al.* 1997; Vanaporn *et al.* 2008), was significantly increased ($P < 0.05$) at 4% NaCl in all strains. *E. coli* O157:H7 gene expression level for the *rpoE* was relatively similar to that of *E. coli* O26 (Fig. 1), indicating that NaCl increased *rpoE* expression. However, it was not correlated to the increased heat resistance of *E. coli* O157:H7. This result may be due to *E. coli* cells being too sensitized by NaCl to be protected by *rpoE*.

When *E. coli* cells were exposed to various concentration of NaCl, the length of *E. coli* O157:H7 cells were shorter in 4% NaCl compared with the length in 0% NaCl. This is similar to the result of a study by Koch (1984), except that the length of *E. coli* O111 were longer in 4% NaCl than in 0% NaCl. A study by Hazeleger *et al.* (2006) also showed that *L. monocytogenes* was elongated after exposure to NaCl. For *E. coli* O26, no change in the cell morphology was observed as NaCl concentration increased (Fig. 2). This result indicates that the effect of NaCl on the cell morphology of *E. coli* is strain-dependent.

In conclusion, the effect of NaCl on the thermal resistance and cell morphology of *E. coli* strains was strain-dependent and higher expression of *proV* by *E. coli* O157:H7 may cause increased thermal resistance. Hence, *proV* inhibitors could be developed through further study or NaCl concentration could be reduced in food processing to suppress *proV* expression.

ACKNOWLEDGMENT

This research was supported by the Sookmyung Women's University Research Grants 1-1303-0162.

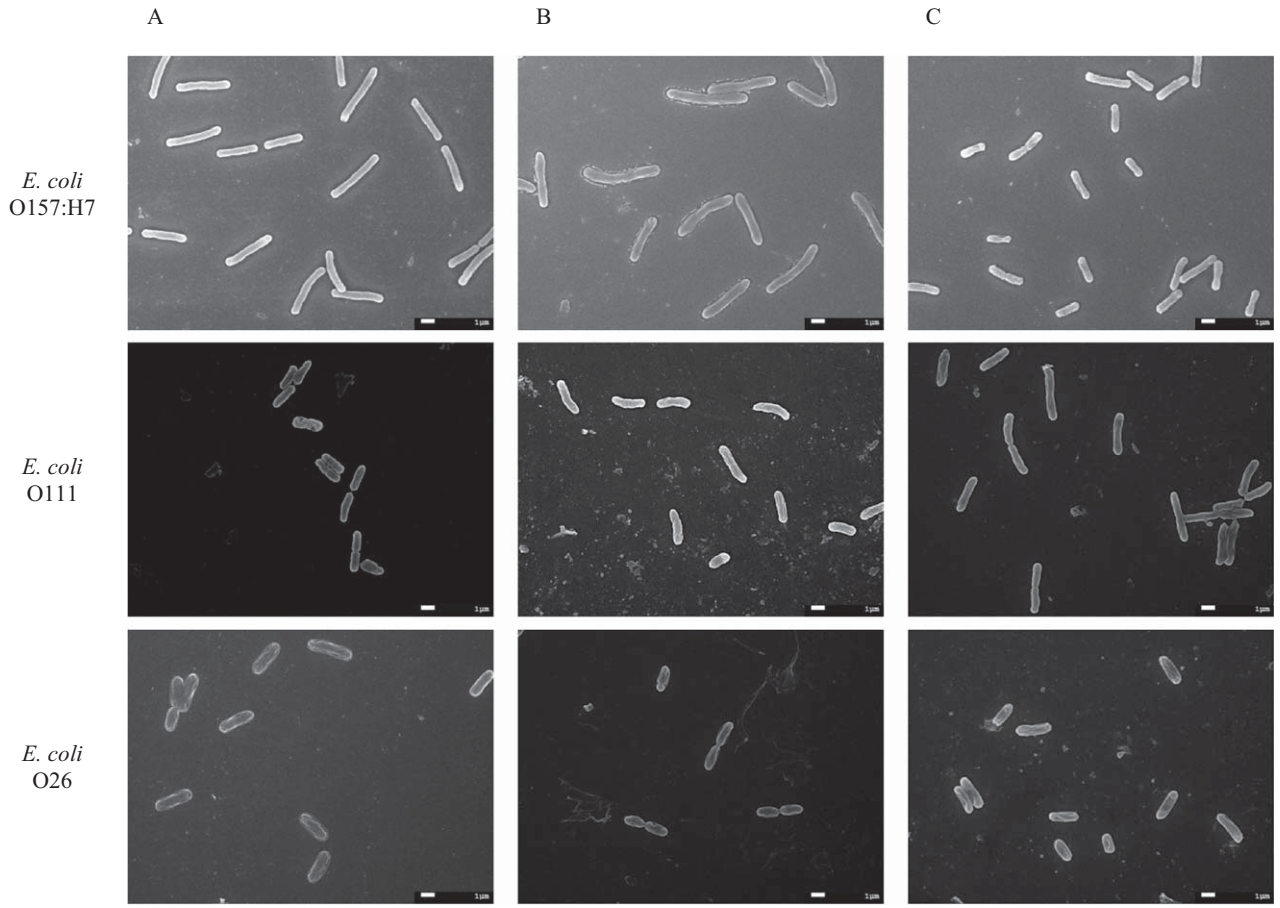

FIG. 2. SCANNING ELECTRON MICROSCOPY IMAGES OF NACL-HABITUATED AND NONHABITUATED *ESCHERICHIA COLI* O157:H7, *E. COLI* O111 AND *E. COLI* O26; (A) 0% NACL, (B) 2% NACL AND (C) 4% NACL

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