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Physicochemical, Mechanical, and Molecular Properties of Nonlysogenic and P22-Lysogenic *Salmonella* Typhimurium Treated with Citrus Oil

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ABSTRACT

The aim of this study was to evaluate the phenotypic and genotypic properties of nonlysogenic *Salmonella* Typhimurium (ST^{P22-}) and lysogenic *Salmonella* Typhimurium (ST^{P22+}) in the presence of sublethal concentrations (SLC_{2D}) of citrus essential oils (CEOs), which were used to evaluate antimicrobial susceptibility, cell surface hydrophobicity, autoaggregation ability, bacterial motility, lysogenic conversion, gene expression patterns, and antibiofilm formation. The SLC_{2D} values of non–heat-treated (N-CEO) and heat-treated (H-CEO) CEO in an autoclave at 121°C for 20 min were 2.0 to 2.1 mg/ml against ST^{P22-} and 1.7 to 1.9 mg/ml against ST^{P22+}. The rates of injured ST^{P22-} and ST^{P22+} cells treated with SLC_{2D} of N-CEO and H-CEO ranged from 67 to 83%. The hydrophobicity and autoaggregation were decreased to 2.5 and 19.5% for ST^{P22-} and 4.7 and 21.7% for ST^{P22+}, respectively, in the presence of N-CEO. A noticeable reduction in the swarming motility was observed in ST^{P22-} with N-CEO (14.5%) and H-CEO (13.3%). The numbers of CEO-induced P22 were 5.40 log PFU/ml for N-CEO and 5.65 log PFU/ml for H-CEO. The relative expression of *hilA, hilC, hilD, invA, invC, invE, invF, sirA,* and *sirB* was down-regulated in ST^{P22-} and ST^{P22+} with N-CEO and H-CEO. The numbers of adherent ST^{P22-} and ST^{P22+} were effectively reduced by more than 1 log in the presence of CEO. These results suggest that CEO has potential to be used to control bacterial attachment, colonization, and invasion.

Salmonella enterica serovar Typhimurium is among the leading causes of acute gastroenteritis in humans, which occurs through consumption of contaminated foods *(9)*. The *Salmonella* infection process is highly complex and is primarily divided into three phases: adherence, colonization, and invasion *(13)*. The bacterial adherence plays a primary role in the enteropathogenesis of *Salmonella* Typhimurium *(13)*. Therefore, the adherence phase can be a critical control point in early intervention strategies to prevent infectious diseases. The bacterial cell surface (autoaggregation and hydrophobicity) and mechanical (swimming and swarming motility) properties are highly correlated with the adhesion ability of pathogens to the host epithelial cells *(26, 31),* which eventually lead to bacterial colonization and invasion. In addition, *Salmonella* pathogenicity island 1 is involved in the infection process though the type III secretion system during the preinvasion phase *(10, 37)*. The expression of the type III secretion system–related genes involved in invasion and intracellular survival is induced in the presence of transcriptional regulator proteins such as HilA, HilC, HilD, InvA, InvC, InvF, SirA, and SirB *(11, 27)*.

The prevention and control of *Salmonella* infections in humans continues to be a high priority in food safety and biomedical research. Over the last few decades, clinical research efforts have been directed toward the development of new, effective, and safe antimicrobials against bacterial infections. Essential oils have long been used in herbal medicines, cosmetics, and foods due to their antibacterial, antifungal, and antiviral activities *(7, 35)*. Recently, the increased prevalence of antibiotic resistant bacteria and consumer demand for green products have led to considerable research attention on naturally occurring antimicrobial agents *(19)*. Citrus essential oils (CEOs) are generally recognized as safe by the U.S. Food and Drug Administration, consisting of linalool (20.2%), decanal (18.0%), geranial, α-terpineol (5.8%), valencene (5.2%), neral (5.0%), dodecanal (4.1%), citronellal (3.9%), and limonene (0.3%) *(22)*. CEOs have been well examined for their antimicrobial activity against *Campylobacter* spp., *Listeria* spp., *Salmonella* spp., and methicillin-resistant *Staphylococcus aureus (21–23)*. To the best of our knowledge, however, there has been no research conducted to examine the antimicrobial activity of CEO against lysogenic bacteria carrying integrated prophage that can convert to virulent bacteriophage. Therefore, the objective of this study was to evaluate the phenotypic and genotypic changes of *Salmonella enterica* serovar Typhimurium ATCC 19585 ($P22^-$) and *Salmonella* Typhimurium ATCC 23555 ($P22^+$) exposed to sublethal concentrations (SLC_{2D}) of non–heat-treated CEOs

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(N-CEOs) and heat-treated CEOs (H-CEOs) by measuring antimicrobial activity, hydrophobicity, autoaggregation, bacterial motility, lysogenic conversion, gene expression patterns, and antibiofilm formation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains of *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 19585 without P22 (ST^{P22-}) and *Salmonella* Typhimurium ATCC 23555 carrying P22 (ST^{P22+}) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), which showed similar survival and growth characteristics. The strains were cultivated in Luria-Bertani broth or on agar plates (Difco, BD, Sparks, MD) at 37°C for 20 h and collected by centrifugation at 3,000 × *g* for 20 min at 4°C. The collected cells were washed with 0.1% sterile buffered peptone water (BPW) and then diluted to approximately 10^8 CFU/ml.

Preparation of citrus oil. Terpeneless cold-pressed Valencia citrus oil was kindly provided by Firmenich Citrus Center (Safety Harbor, FL). A stock solution was prepared by dissolving CEO in 50% dimethylsulfoxide (wt/vol) and sterilized by filtration through 0.2-μm-pore-size dimethylsulfoxide-safe Acrodisc filter (Pall Corporation, Port Washington, NY). The sterilized stock solution was divided into two aliquots, one of which was autoclaved at 121°C for 20 min to evaluate the heat stability of CEO. Treatments included the nontreated control containing no CEO (CON), N-CEO, and H-CEO.

Antimicrobial susceptibility assay. The antimicrobial activity patterns were determined by a concentration-kill curve of ST^{P22-} and ST^{P22+} (10^6 CFU/ml each) cultured in Luria-Bertani containing different concentrations (0 to 8.8 mg/ml) of CEO at 37°C for 18 h. The dose-response curves were analyzed using the Nonlinear Curve Fitting Function of Microcal Origin 7.5 (Microcal Software Inc., Northampton, MA) and we determined that the SLC_{2D} microbial numbers were reduced by less than 2 log compared with the control *(40)*. The SLC_{2D} values of N-CEO and H-CEO were 1.97 and 2.07 mg/ml, respectively, against ST^{P22-} and 1.70 and 1.90 mg/ml against ST^{P22+}, which were used as sublethal stress conditions for the following assays.

Determination of antimicrobial-injured cells. The antimicrobial CEO-induced injury was evaluated according to the overlay method *(12)*. The sublethally injured *Salmonella* Typhimurium cells were sensitive to xylose lysine desoxycholate (XLD) agar. The nontreated control and CEO-treated cells at SLC_{2D} cultured at 37°C for 18 h were serially (1:10) diluted with 0.85% NaCl solution, plated on Trypticase soy agar (TSA) and XLD overlaid on TSA agar, and incubated at 37°C for 24 to 48 h. The antimicrobial-induced injury rate was estimated by the difference between the cell numbers obtained from TSA and XLD agar.

Cell surface hydrophobicity assay. The bacterial cell surface hydrophobicity was determined using a bacterial affinity to hydrocarbon assay *(28)* with minor modifications. The bacterial cells grown at the control (no treatment) and predetermined SLC_{2D} of CEOs at 37°C for 18 h were centrifuged at 3,000 × *g* for 20 min and resuspended in phosphate-buffered saline (PBS; pH 7.2) to OD_{570} values of 0.5 (H_{t0}). Each suspension (2 ml) was mixed with 1 ml of *n*-hexadecane and let stand for 5 min. The aqueous phase was measured at 570 nm (H_{t5}) using a Multiskan microplate reader (Thermo Fisher Scientific Inc., Fair Lawn, NJ). The hydrophobicity was estimated following the equation:

$$\text{Hydrophobicity } (\%) = (1 - H_{t5}/H_{t0}) \times 100$$

Autoaggregation assay. The interactions between bacteria cell surfaces were determined by the autoaggregation assay *(5)* with minor modifications *(40)*. The bacterial cells grown in the control and SLC_{2D} of CEOs at 37°C for 18 h were centrifuged at 3,000 × *g* for 20 min and diluted with PBS (pH 7.2) and adjusted to OD_{570} values of 0.5 (A_{t0}). The suspensions (3 ml each) were incubated at 37°C for 2 h. The upper suspensions were measured at 570 nm (A_{t2}) using a Multiskan microplate reader (Thermo Fisher Scientific Inc.). The ability of bacterial cells to aggregate was estimated as follows:

$$\text{Autoaggregation } (\%) = (1 - A_{t2}/A_{t0}) \times 100$$

Bacterial motility assay. Bacterial motilities were determined according to the swimming and swarming motility assays *(20)* with slight modifications. The bacterial cells cultured in the control and predetermined SLC_{2D} of CEOs at 37°C for 18 h were resuspended in PBS and adjusted to OD_{570} values of 0.5. Each culture (3 μl) was stabbed into the center of soft agar plates for swimming motility (1% tryptone, 0.7% NaCl, and 0.25% agar) and swarming motility (1% tryptone, 0.5% NaCl, 0.5% glucose, and 0.45% agar). The swimming and swarming plates were incubated at 22 and 28°C for 24 h, respectively. The diameters of motility zones on swimming and swarming plates were measured. The swimming and swarming motilities of treated cells were expressed as percent compared with the control.

Determination of antimicrobial-induced prophage. The numbers of lytic phages were enumerated to evaluate the effect of CEOs on the lysogenic conversion of $P22^+$. The bacterial cells grown in the control (no treatment) and predetermined SLC_{2D} at 37°C for 18 h were centrifuged at 3,000 × *g* for 20 min and filtered through a 0.2-μm-pore-size sterilized filter. The mitomycin C (1 μg/ml) pretreated cells were used as a positive control for phage induction. The collected supernatants were serially (1:10) diluted with 0.1% BPW to enumerate the lytic phages using a soft agar overlay method *(1)*. Appropriate dilutions were gently mixed with *Salmonella* Typhimurium host cells in 0.5% Luria-Bertani soft agar and then poured onto the surface of prewarmed 1.5% Luria-Bertani agar. The plates were incubated at 37°C for 24 h. The lytic phages induced at the control (BP_c) and treatments (BP_t) were enumerated and expressed as PFUs. The number of antimicrobial-induced phages was estimated as $BP_t - BP_c$. The mortality rate and burst size were calculated as follows *(39)*:

$$\text{Mortality rate } (\%) = [(ST_c - ST_t)/S_c] \times 100$$

$$\text{Burst size } (\%) = (BP_t - BP_c)/(ST_c - ST_t)$$

where ST_c and ST_t are the numbers of *Salmonella* Typhimurium cells at the control and treatment, respectively.

RNA extraction and cDNA synthesis. The extraction of RNA was carried out according to the protocol of ZR Bacterial RNA MiniPrep kit (Zymo Research Corp., Irvine, CA). The cells grown in the control and SLC_{2D} of CEOs were centrifuged at 3,000 × *g* for 20 min, resuspended with 800 μl of RNA Lysis Buffer in a ZR BashingBead Lysis Tube, homogenized twice for 30 s at a speed setting of 5.0 m/s using a bead beater (FastPrep-24 System Kit, MP Biomedicals, Solon, OH), and then centrifuged at 12,000 × *g* for 1 min. The supernatant (400 μl) was transferred to a Zymo-Spin IIIC Column in a collection tube and centrifuged at 8,000 × *g* for 30 s. The flow-through fraction was mixed with

TABLE 1. *Molecular functions and primer sequences of target genes used in quantitative reverse transcriptase PCR analysis for* Salmonella *Typhimurium*

Gene	Molecular function	Primer sequence	Accession no.
50S ribosomal protein L5	Reference gene	F:GTAGTACGATGGCGAAACTGC R:CTTCTCGACCCGAGGGACTT	M36266
hilA	Invasion protein regulator	F:TATCGCAGTATGCGCCCTTT R:CAAGAGAGAAGCGGGTTGGT	U25352
hilC	Invasion regulatory protein	F:AATGGTCACAGGCTGAGGTG R:ACATCGTCGCGACTTGTGAA	AF134856
hilD	Invasion regulatory protein	F:CTCTGTGGGTACCGCCATTT R:TGCTTTCGGAGCGGTAAACT	U21676
invA	Invasion protein	F:CGCGCTTGATGAGCTTTACC R:CTCGTAATTCGCCGCCATTG	U43237
invC	Type III secretion system ATPase	F:GCTGACGCTTATCGCAACTG R:GGCGGTGCGACATCAATAAC	U08279
invE	Invasion protein	F: CGAATGACGCCAGCTGTTC R:TGCGTCAGGCGTCGTAAA	M90714
invF	Invasion regulatory protein	F:TCGCCAAACGTCACGTAGAA R:CATCCCGTGTATAACCCCCG	U08280
sirA	Transcriptional regulator	F:TCCAGCTACTTTCGCAGCAA R:AACACGTTGTAACGCGGTTG	U88651
sirB	Transcription factor	F:AGGCGCGTTTTATCGCTTTC R:CCAGACTGACCAGACGTTCC	AF134855

320 μl of 95% ethanol, transferred to a Zymo-Spin IC Column in a collection tube, and centrifuged at 12,000 × *g* for 30 s. The Zymo-Spin IC Column was rinsed with 400 μl of RNA wash buffer at 12,000 × *g* for 1 min and incubated at 37°C for 20 min with 30 μl of DNase I containing 3 μl of RNase-free DNase I, 3 μl of 10× reaction buffer, and 24 μl of RNA wash buffer. After incubation, the Zymo-Spin IC Column was treated with 400 μl of RNA prep buffer and centrifuged at 12,000 × *g* for 30 s. The column was rinsed twice with 800 μl of RNA wash buffer and centrifuged at 12,000 × *g* for 2 min to remove the remaining wash buffer. RNA was eluted by adding DNase/RNase-free water (25 μl) in the Zymo-Spin IC Column at 10,000 × *g* for 30 s and quantified using a NanoDrop spectrophotometer (Thermo Scientific Inc., West Palm Beach, FL). The cDNA synthesis was performed according to the protocol of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The eluted RNA (1 μg) was mixed with 4 μl of 5× qScript cDNA SuperMix containing the optimized concentration of $MgCl_2$, deoxynucleoside triphosphate, RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer, and stabilizers and then incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

Quantitative reverse transcriptase PCR assay. The mixture containing 10 μl of PerfeCTa SYBR Green FastMix, 2 μl of each primer (100 nM), 2 μl of cDNA (10 ng), and 4 μl of RNase-free water was amplified using an Eco RT PCR system (Illumine, San Diego, CA), which was denatured at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s. The custom-synthesized oligonucleotides (Erofins MWG Operon, Huntsville, AL) used as primers to target conserved regions of *Salmonella* Typhimurium are listed in Table 1. The relative expression levels of genes were calculated by the comparative method *(16)*. The cycle threshold (C_T) values of target genes in treated *Salmonella* Typhimurium cells were compared with the C_T values obtained from the control. The housekeeping gene, 50S ribosomal protein L5, was used as the reference gene for normalization of target gene expression.

Biofilm formation assay. The ability of ST^{P22-} and ST^{P22+} to adhere to cell surfaces was evaluated in the control (CON) and the predetermined SLC_{2D} of CEOs (N-CEO and H-CEO) using a 24-well flat-bottomed polystyrene microtiter plate (BD Falcon, San Jose, CA). ST^{P22-} or ST^{P22+} was inoculated at approximately 10^6 CFU/ml in the CON, N-CEO, and H-CEO. After 24 h of incubation at 37°C, each well was rinsed twice with 0.1% BPW to eliminate loosely attached cells. The adherent cells were collected by using sterile cotton swabs, which were immersed in 1 ml of 0.1% BPW and vortexed vigorously to suspend adhered cells. The suspensions were serially (1:10) diluted with 0.1% BPW. Proper dilutions were plated on TSA and incubated at 37°C for 24 to 48 h to enumerate the biofilm cells.

Statistical analysis. The assays of antimicrobial susceptibility, hydrophobicity, autoaggregation, antimicrobial-induced injury, antibiofilm formation, and lysogenic conversion were conducted in duplicate for three replicates. Motility and quantitative reverse transcriptase PCR analyses were carried out in five trials of each replicate. All data were analyzed using the Statistical Analysis System software (SAS Institute Inc., Cary, NC). The general linear model and Fisher's least significant difference procedures were used to determine significant differences among treatments at $P < 0.05$.

RESULTS AND DISCUSSION

This study demonstrates the phenotypic and genotypic changes in ST^{P22-} and ST^{P22+} when treated with CEOs in association with adhesion, colonization, and invasion characteristics. A comprehensive understanding of the relationships among physicochemical, mechanical, and molecular properties of *Salmonella* Typhimurium is needed to investigate the possibility of using CEOs at the cellular level.

Antimicrobial susceptibility, physicochemical property, and motility of *Salmonella* Typhimurium grown at the SLC_{2D} of CEOs. The CEO-induced cell injury was evaluated on nonselective (TSA) and selective XLD agar plates by using the overlay method (Table 2). The rates

TABLE 2. *Physicochemical properties and mechanical behaviors of* Salmonella *Typhimurium treated with citrus essential oils (CEOs)*

Strain[a]	Treatment[b]	Injured-cell rate (%)	Hydrophobicity (%)	Autoaggregation (%)	Swimming motility (%)	Swarming motility (%)
ST^{P22-}	CON	21.79 ± 6.41 D[c]	5.82 ± 0.39 AB	24.75 ± 1.87 A	100[d]	100
	N-CEO	70.34 ± 10.57 BC	2.51 ± 0.81 D	19.47 ± 2.63 C	94.07 ± 5.59 A	49.65 ± 4.28 B
	H-CEO	67.92 ± 12.40 C	2.07 ± 0.86 D	20.20 ± 2.47 C	88.52 ± 9.96 A	53.38 ± 3.41 B
ST^{P22+}	CON	15.29 ± 4.71 D	6.46 ± 0.60 A	24.24 ± 2.39 AB	100	100
	N-CEO	78.40 ± 9.84 AB	4.74 ± 0.88 C	21.65 ± 1.82 BC	14.53 ± 1.84 B	53.50 ± 4.32 B
	H-CEO	82.46 ± 10.34 A	5.01 ± 0.52 BC	21.30 ± 1.37 BC	13.25 ± 1.09 B	62.27 ± 5.21 A

[a] ST^{P22-}, *Salmonella* Typhimurium without P22; ST^{P22+}, *Salmonella* Typhimurium with P22.
[b] CON, nontreated control; N-CEO, non–heat-treated citrus essential oil; H-CEO, heat-treated citrus essential oil.
[c] Means with different letters within a column (A through D) are significantly different at $P < 0.05$.
[d] Mortality values are normalized to untreated control (100%).

of injured ST^{P22-} and ST^{P22+} cells were significantly increased, ranging from 67 to 83% by the CEO treatments (N-CEO and H-CEO). This result suggests that the CEO could induce bacterial injury of both ST^{P22-} and ST^{P22+} cells. The rates of injured cells were not different between N-CEO and H-CEO treatments. The observation that there were no significant differences in antimicrobial activity between N-CEO and H-CEO indicates that the components in CEO might be thermally stable. This confirms previous reports that the chemical components of CEOs and natural extracts, including limonene, terpinene, myrcene, kaempferol, quercetin, myricetin, carvacrol, and *p*-cymene, are very heat stable *(33)*. The hydrophobicity and autoaggregation ability were measured to understand the physicochemical surface properties of ST^{P22-} and ST^{P22+} treated with SLC_{2D} of CEOs (Table 2). ST^{P22-} showed the lowest hydrophobicity for N-CEO (2.5%) and H-CEO (2.1%). A noticeable change in the hydrophobicity was detected in ST^{P22-} with N-CEO (4.1%) and H-CEO (5.0%) when compared with the control (6.5%). Similar to the hydrophobicity patterns, the least autoaggregation ability was observed in ST^{P22-} with N-CEO (19.5%) and H-CEO (20.2%). The cell surface hydrophobicity and autoaggregation ability of both ST^{P22-} and ST^{P22+} cells were decreased in the presence of CEOs, which can be potentially responsible for the inhibition of initial bacterial adhesion to the epithelial cell surface. It has been previously reported that cell surface hydrophobicity and autoaggregation were positively correlated with the bacterial attachment *(29, 31)*. Therefore, these physicochemical factors can be used as indicators to assess bacterial adhesion to the epithelial surface in the human gastrointestinal tract *(29, 31)*. The motility phenotypes of ST^{P22-} and ST^{P22+} treated with SLC_{2D} of CEOs were examined through liquid-based movement (swimming motility) and solid-based movement (swarming motility) on semisolid agar plates (Table 2). CEOs effectively reduced the swimming motility of ST^{P22+}. The swimming motility of ST^{P22+} was significantly reduced by 14.5 and 13.3%, respectively, with N-CEO and H-CEO. The least changes in swimming motility were observed in ST^{P22-} with N-CEO (94.1%) and H-CEO (88.5%). The swarming motility was relatively high in ST^{P22+} with H-CEO (62.3%) when compared with other treatments, showing 49.7% in ST^{P22-} with N-CEO, 53.4% in ST^{P22-} with H-CEO, and 53.5% in ST^{P22+} with N-CEO. The flagella-mediated swimming motility is mainly involved in bacterial translocation to evade the host immune system *(2)*. The surface-associated swarming motility plays an important role in bacterial colonization via type IV pili and quorum sensing, leading to biofilm formation *(24, 36)*. The decrease in swimming and swarming motility indicates that CEO can effectively control the bacterial colonization and invasion. The influence of CEOs on prophage induction was evaluated in ST^{P22+} cells, as shown in Table 3. The prophage P22 was spontaneously released at 4.37×10^4 PFU/ml from ST^{P22+} cells (data not shown). The number of mitomycin C–induced P22 was 11.36 log PFU/ml, while relatively low numbers of P22 were induced by N-CEO (5.40 log PFU/ml) and H-CEO (5.65 log PFU/ml). The numbers of ST^{P22+} cells were reduced by more than 2 log compared with the control, showing more than 99% of mortality. CEOs mostly contributed to the bacterial mortality but less to the prophage induction. This observation indicates that the CEOs are more likely to disrupt cell membrane permeability *(38)* rather than inhibit DNA synthesis, leading to the SOS induction by RecA-mediated cleaves of c2 repressor in ST^{P22+} cells *(14)*.

TABLE 3. *Induction of P22 prophage in* ST^{P22+} *treated with citrus essential oils (CEOs)*

	Salmonella Typhimurium ($P22^+$)		
Treatment[a]	Induced phage (PFU/ml)[b]	Bacterial mortality (%)[c]	Burst size[d]
MMC	2.28×10^{11}	94.75 (1.31 ± 0.19)[e]	86.45
N-CEO	2.54×10^5	99.31 (2.23 ± 0.33)	0
H-CEO	4.49×10^5	99.43 (2.32 ± 0.30)	0

[a] MMC, mitomycin C (1 μg/ml); N-CEO, non–heat-treated citrus essential oil; H-CEO, heat-treated citrus essential oil.
[b] The number of antimicrobial-induced phages is estimated as ($BP_t - BP_c$). BP_c and BP_t indicate the numbers of lytic phages induced at the control and treatment, respectively.
[c] Mortality rate (%) = $(ST_c - ST_t)/S_c \times 100$. ST_c and ST_t are the numbers of *Salmonella* Typhimurium cells at the control and treatment, respectively.
[d] Burst size = $(BP_t - BP_c)/(ST_c - ST_t)$.
[e] The value in parentheses is the reduction number of *Salmonella* Typhimurium ($P22^+$) cells (log CFU per milliliter) compared with the control.

TABLE 4. *Relative gene expression in* Salmonella *Typhimurium grown in SLC_{2D} of citrus essential oils (CEOs)*

Strain[a]	Treatment[b]	Fold change in gene expression								
		hilA	*hilC*	*hilD*	*invA*	*invC*	*invE*	*invF*	*sirA*	*sirB*
ST^{P22-}	N-CEO	−1.42 d BC[c]	−0.56 c C	−2.24 b B	−0.93 d BC	−5.70 a A	−5.79 a A	−0.36 c C	−2.24 bc B	−1.11 b BC
	H-CEO	−3.27 c C	−2.03 b D	−3.61 a C	−3.38 c C	−3.40 b C	−5.71 a A	−4.44 b B	−2.83 ab C	−1.17 b E
ST^{P22+}	N-CEO	−5.73 b B	−3.37 a C	−3.98 a C	−8.57 a A	−1.60 c D	−0.66 b D	−8.46 a A	−3.90 a C	−3.48 a C
	H-CEO	−7.38 a B	−2.41 b F	−3.45 a E	−6.84 b BC	−5.92 a D	−6.24 a CD	−8.42 a A	−1.68 c F	−2.04b F

[a] ST^{P22-}, *Salmonella* Typhimurium without P22; ST^{P22+}, *Salmonella* Typhimurium with P22.
[b] N-CEO, non–heat-treated citrus essential oil; H-CEO, heat-treated citrus essential oil.
[c] Means with different letters within a column (a through d) and a row (A through F) are significantly different at $P < 0.05$.

Differential gene expression and antibiofilm ability of *Salmonella* Typhimurium grown at the SLC_{2D} of CEOs. The relative expression patterns of genes responsible for bacterial invasion were examined in both ST^{P22-} and ST^{P22+} treated with SLC_{2D} of CEOs (Table 4). The transcript levels of all genes tested were decreased in both ST^{P22-} and ST^{P22+} with N-CEO and H-CEO treatments. The relative expression levels of *invC* and *invE* genes were significantly decreased to more than fivefold in ST^{P22-} with N-CEO compared with other genes. The *invE* gene was highly decreased to 5.7-fold in ST^{P22-} with H-CEO. The *invA* and *invF* genes were significantly repressed by more than sixfold in ST^{P22+} with N-CEO and H-CEO. Most genes were more extensively down-regulated in ST^{P22+} with N-CEO and H-CEO than ST^{P22-}. The results suggest that the CEOs can negatively affect transcriptional regulatory proteins responsible for bacterial adhesion and invasion. The expression of transcriptional regulators encoded within *Salmonella* pathogenicity island 1 (*prg*/*org*, *inv*/*spa*, and *sic*/*sip* operons) is essential for bacterial attachment and invasion, which eventually results in inflammatory diseases *(8, 15)*. The BarA/SirA two-component regulatory system contributes to virulence, motility, and resistance in *Salmonella enterica (34)*. SirA activated by *crp* and *cya* genes positively controls the transcription of *hilA* and *hilC*, which acts as an initial effector at the top of bacterial invasion pathway *(11, 34)*. Therefore, the expression of *sirA* in *Salmonella* Typhimurium leads to surface adhesion and biofilm formation *(30)*. The AraC/XylS-type regulators (HilC and HilD) positively modulate the expression of *hilA* as transcription activators binding to the upstream repression sites *(6, 8, 18)*. HilD also controls the flagella regulon (FliZ) *(3)*, implying that the motility decrease in ST^{P22-} and ST^{P22+} treated with CEOs can be attributed to the repression of *hilD*. HilA belonging to a ToxR/OmpR-type regulator activates *prg*/*org* and *inv*/*spa* operons, which encode *prgH* and *invF*, respectively *(17)*. PrgH is necessary for the entry of *Salmonella* Typhimurium into the epithelial cells *(6)*. Darwin and Miller *(4)* reported that InvF with the cofactor SicA activates the expression of *sipB*, *sipC*, *sipD*, *sigD*, and *sopE*. The antibiofilm activities of CEOs on the growth of both ST^{P22-} and ST^{P22+} were evaluated at 37°C for 24 h (Fig. 1). The numbers of adherent ST^{P22-} and ST^{P22+} cells for the control were increased up to 5.88 and 5.68 CFU/ml, respectively, which were effectively reduced by more than 1 log with the N-CEO and H-CEO treatments. The inhibitory activity of CEOs on the biofilm formation corresponded to the hydrophobic interaction, autoaggregation, and bacterial motility. This observation confirms that physicochemical properties and bacterial motility play an important role in the attachment of ST^{P22-} and ST^{P22+} cells to the host cell surfaces, initiating bacterial infectious process. Biofilm formation is positively correlated with autoaggregation and bacterial motility *(2, 25, 32)*.

In conclusion, the CEO could effectively reduce the numbers of adherent ST^{P22-} and ST^{P22+} cells in association with the decreased hydrophobic interaction, autoaggregation ability, and bacterial motility. The most significant findings in this study were that (i) the hydrophobicity autoaggregation was positively correlated with the initial adherence of ST^{P22-} and ST^{P22+} cells to the surface, (ii) the swarming motility was more positively related to the bacterial colonization than the swimming motility, (iii) the CEO-induced lysogenic conversion occurred at low frequency, and (iv) the expression of adhesion-related genes were repressed in ST^{P22-} and ST^{P22+} cells exposed to SLC_{2D} of CEOs, resulting in the effective inhibition of a *Salmonella* invasion-mediated cascade. These physicochemical, behavioral, and molecular properties can be used as selection tools to

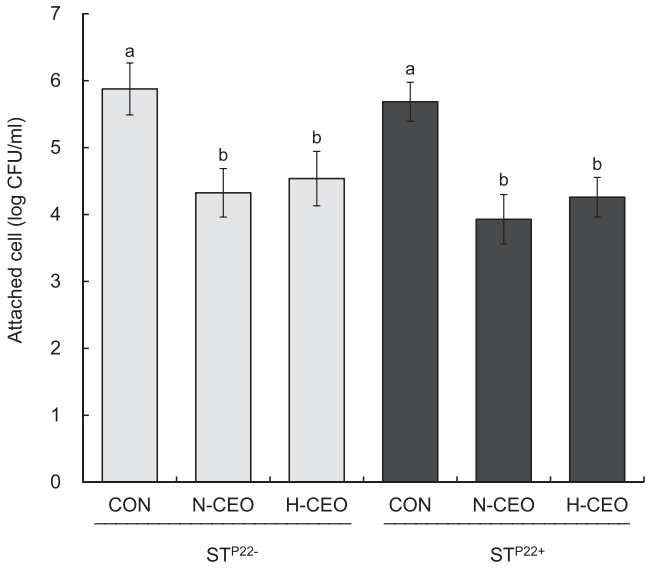

FIGURE 1. *Antibiofilm activity of citrus essential oils (CEOs) against nonlysogenic* Salmonella *Typhimurium (ST^{P22-}) and lysogenic* Salmonella *Typhimurium (ST^{P22+}). Different letters (a and b) on the bars are significantly different at* $P < 0.05$.

evaluate the bacterial adhesion, colonization, and invasion to the host epithelial cells. This study provides primary insight into the possibility of using CEOs for controlling *Salmonella* infection in human and animals. However, further study is needed to understand the mode of antimicrobial action of CEOs against pathogens when exposed to complex gut environmental conditions. Such study at the cellular level is currently under investigation in our laboratory.

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