Journal of Food Protection, Vol. 75, No. 8, 2012, Pages 1418–1428
doi:10.4315/0362-028X.JFP-11-427

Biofilm Formation by Shiga Toxin–Producing *Escherichia coli* O157:H7 and Non-O157 Strains and Their Tolerance to Sanitizers Commonly Used in the Food Processing Environment†

RONG WANG,* JAMES L. BONO, NORASAK KALCHAYANAND, STEVEN SHACKELFORD, AND DAYNA M. HARHAY

U.S. Department of Agriculture, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska 68933-0166, USA

MS 11-427: Received 21 September 2011/Accepted 26 March 2012

ABSTRACT

Shiga toxin–producing *Escherichia coli* (STEC) strains are important foodborne pathogens. Among these, *E. coli* O157:H7 is the most frequently isolated STEC serotype responsible for foodborne diseases. However, the non-O157 serotypes have been associated with serious outbreaks and sporadic diseases as well. It has been shown that various STEC serotypes are capable of forming biofilms on different food or food contact surfaces that, when detached, may lead to cross-contamination. Bacterial cells at biofilm stage also are more tolerant to sanitizers compared with their planktonic counterparts, which makes STEC biofilms a serious food safety concern. In the present study, we evaluated the potency of biofilm formation by a variety of STEC strains from serotypes O157:H7, O26:H11, and O111:H8; we also compared biofilm tolerance with two types of common sanitizers, a quaternary ammonium chloride–based sanitizer and chlorine. Our results demonstrated that biofilm formation by various STEC serotypes on a polystyrene surface was highly strain-dependent, whereas the two non-O157 serotypes showed a higher potency of pellicle formation at air-liquid interfaces on a glass surface compared with serotype O157:H7. Significant reductions of viable biofilm cells were achieved with sanitizer treatments. STEC biofilm tolerance to sanitization was strain-dependent regardless of the serotypes. Curli expression appeared to play a critical role in STEC biofilm formation and tolerance to sanitizers. Our data indicated that multiple factors, including bacterial serotype and strain, surface materials, and other environmental conditions, could significantly affect STEC biofilm formation. The high potential for biofilm formation by various STEC serotypes, especially the strong potency of pellicle formation by the curli-positive non-O157 strains with high sanitization tolerance, might contribute to bacterial colonization on food contact surfaces, which may result in downstream product contamination.

Shiga toxin–producing *Escherichia coli* (STEC) strains of various serotypes are important foodborne pathogens that pose a serious public health concern, resulting in significant financial burden. STEC strains have been implicated in numerous outbreaks, with symptoms ranging from bloody diarrhea to other, more severe, diseases such as hemolytic uremic syndrome (HUS), a life-threatening complication that is the major cause of kidney failure for children younger than the age of 5 years *(11)*. Cattle are the principal animal reservoir of these zoonotic pathogens, and foodborne outbreaks of illness have been associated with the consumption of ground beef, dairy products, vegetables, and fruit juices, etc. *(20–22)*.

Since it was first identified in 1982, STEC serotype O157:H7 has become the most commonly identified STEC serotype, causing multiple clinical diseases and foodborne outbreaks in North America, Europe, and Asia. Serotype O157:H7 infection can cause bloody diarrhea, HUS, and thrombotic thrombocytopenic purpura, etc. It has been estimated that *E. coli* O157:H7 is responsible for over 73,000 cases of illness each year in the United States *(8)*. Meanwhile, numerous outbreaks associated with the non-O157 serotypes also have contributed to the public burden of human infections and clinical diseases. Despite increased isolation of non-O157 STEC strains from patients, outbreaks, and environmental sources *(32, 33),* the public health significance of these pathogens has not been well investigated due to diagnostic limitations and inadequate surveillance. According to studies at the Centers for Disease Control and Prevention (CDC), approximately 70% of the non-O157 STEC infections that emerged from 1983 to 2002 were caused by one of six major serotypes, which are now referred to as ''the big six,'' including O26, O45, O103, O111, O121, and O145. It was reported that these six STEC serotypes, collectively, caused more human infections in the United States in 2010 than did STEC O157:H7; therefore, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) has announced the intention to carry out an enforceable program to give these non-O157

* Author for correspondence. Tel: 402-762-4228; Fax: 402-762-4149; E-mail: rong.wang@ars.usda.gov.

† The U.S. Department of Agriculture is an equal opportunity provider and employer. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

STECs the same regulatory scrutiny as STEC O157:H7 in the national's beef supply *(45)*. Among these six non-O157 serotypes, O26 is the most common non-O157 STEC isolated from specimens submitted to CDC for serotyping. O26 was found to be responsible for 40% of all non-O157 and 14% of total STEC-related cases of HUS in Austria and Germany between 1996 and 2003 *(1)*. This serotype also was found to play an important role in the etiology of acute diarrhea in young children *(13, 34)*. Recovery of serotype O26 from cattle and beef products has been reported in different countries *(1, 29, 47)*. On the other hand, serotype O111 is the second most common non-O157 STEC strain isolated from specimens submitted to the CDC. This serotype has been associated with sporadic cases and outbreaks of bloody diarrhea. The first case of community O111 outbreak was reported in Texas in 1999, and further analysis of samples from patients confirmed that STEC O111:H8 was the causative agent of the infection. The largest foodborne outbreak due to STEC O111 in the United States was reported in Oklahoma in 2008; it caused over 340 cases of human infections and severe diarrhea illness *(6)*. In addition, one study reported that O111 was responsible for the majority of HUS cases in the United States *(5)*. O111:H8 and O111:nonmotile strains are the two most frequently isolated O111 serotypes, and these pathogens have been detected in cattle fecal samples and in ground beef products *(42)*.

It has been shown that various STEC serotypes have the ability to attach, colonize, and form biofilms on a wide variety of food contact surfaces commonly used in meat-processing plants as well as on vegetables and meat products *(25, 39)*. Food processing equipment design and surface materials also could affect bacterial attachment and biofilm formation. In addition, bacterial biofilms are usually much more tolerant to sanitizing agents than free-flowing cells of the same species, so it is difficult to completely inactivate biofilms formed on the equipment and in the environment, and the surviving biofilm cells may detach from the surface and contaminate food products. Strong attachment of the STEC biofilms on food surfaces may also affect the efficiency of antimicrobial interventions applied to food products for reducing contamination. Thus, STEC biofilm formation is a serious potential hazard in food hygiene and may become a source of cross-contamination in the food processing environment. Prevention, removal, and inactivation of STEC biofilms, therefore, are critical for improving hygiene, controlling contamination, and enhancing food safety.

Considerable research has been directed at evaluating the impact of STEC biofilms on food safety, as well as understanding the mechanisms and genetic basis for biofilm formation by these pathogens *(3, 11, 25, 27, 38, 39)*. Studies also have investigated the tolerance of STEC cells in biofilms to decontamination reagents *(18, 19, 26, 40)*. However, most of these studies have focused on serotype O157:H7 since it is the serotype most commonly associated with foodborne outbreaks and clinical diseases. In contrast, there are relatively few reports on the ability of non-O157 serotypes to form biofilms *(2, 9, 28, 41)* and to tolerate sanitizers. In the present study, we compared various strains of STEC serotypes O157:H7, O26:H11, and O111:H8 for their ability to form biofilms under different conditions and for the tolerance of their biofilms to sanitizing regents commonly used in the food processing environment.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and curli expression. Ten strains each of STEC O157:H7, STEC O26:H11, and STEC O111:H8 that varied epidemiologically by sources were used for biofilm formation and sanitization study (Table 1). Each isolate was characterized by enzyme-linked immunosorbent assay, using anti-O157, O26, O111, and H7 monoclonal antibodies and multiplex PCR for stx_1, stx_2, *eae, hlyA,* rfb_{O157}, $fliC_{H7}$ rfb_{O26}, $fliC_{H11}$ rfb_{O111}, and $fliC_{H8}$ *(11, 12, 14, 16, 31, 36, 48)*. Additionally, each strain was genotyped for a polymorphism residing within the translocated intimin receptor gene (*tir* 255 T > A) *(3)*. Curli expression of these STEC strains was screened using Congo red indicator (CRI) plates as previously described *(15)*.

All strains were stored at −70°C in Lennox broth (LB; Acumedia Manufacturers, Baltimore, MD) containing 15% glycerol. Prior to use, each strain was streaked from the glycerol stock onto LB agar plates and grown overnight at 37°C. To prepare bacterial broth cultures at stationary phase for biofilm formation and sanitization study, bacteria from a single colony on LB agar plates were inoculated into LB–low salt (LB-LS) broth and incubated overnight (16 to 18 h) at 37°C with orbital shaking at 150 rpm.

Sanitizers. Two types of commercial sanitizers commonly used in the food processing industry were tested in this study. The quaternary ammonium chloride (QAC)–based sanitizer, Vanquish (Total Solutions, Milwaukee, WI), contains a mixture of alkylbenzyldimethylammonium chlorides with various even-numbered alkyl chain lengths as active ingredients. This product is a broad-spectrum disinfectant and sanitizer authorized by the USDA as category D2 for use in meat, poultry, and other food processing plants. Vanquish is also an Environmental Protection Agency–registered pesticide product, and it can be applied to sanitize hard nonporous surfaces of food processing equipment, utensils, dishes, countertops, and other precleaned food contact surfaces without requiring a rinse after its use. Following the manufacturer's instructions, a 1:171 dilution of Vanquish, containing 300 ppm of active ingredients, was used for all QAC treatments throughout the study. The chlorine solution at the final concentration of 200 ppm was prepared in sterile distilled water from commercial germicidal bleach containing 6.15% sodium hypochlorite (Clorox, Oakland, CA), and the chlorine level in the solution was confirmed using High Range Free Chlorine Test Strips (LaMotte Co., Chestertown, MD).

In vitro quantitative assay for biofilm formation on polystyrene microtiter plates. To assess biofilm formation by the crystal violet (CV) staining assay, bacteria strains were grown overnight as described above to reach a cell concentration of approximately 5×10^8 CFU/ml. The overnight culture of each strain was 100-fold diluted in sterile LB-LS broth and added to 96-well flat-bottom polystyrene plates (Costar, Corning, NY) at 200 μl per well. Control wells contained only sterile LB-LS broth. Plates were incubated statically for 24, 48, or 72 h at 22 to 25°C. After incubation, supernatants in each well were gently harvested by aspiration, and bacterial planktonic growth was determined by measuring A_{600} using a microplate reader (Molecular Devices, Sunnyvale, CA). The plates were washed with 200 μl per well of 1× sterile

TABLE 1. *Bacterial strains, virulence genes, and curli expression*

Serotype	Strain	Origin[a]	*stx*$_1$[b]	*stx*$_2$[b]	*eae*[b]	*hlyA*[b]	Curli[c]	Reference/ source[d]
O157:H7	FSIS 10	G		y	y	y	−	MH
	FSIS 11	G		y	y	y	−	MH
	FSIS 53	G	y	y	y	y	−	MH
	FSIS 62	G	y	y	y	y	±	MH
	FSIS 71	G	y	y	y	y	−	MH
	FSIS 78	G	y	y	y	y	−	MH
	USDA 39	H	y	y	y	y	−	ES
	RM 6049	H					−	*7*
	USDA 2	H		y	y	y	−	ES
	TX 909-1	B		y	y	y	−	*4*
O26:H11	10205	H	y	y	y	y	+	IDHW
	DECA 10A	H	y		y	y	±	NFSTC
	7-14 50A	B		y	y	y	++	MARC
	18 B24-2	B	y		y	y	−	MARC
	DEC 10B	H	y		y	y	±	*49*
	DEC 10E	B	y	y	y	y	−	*49*
	CL5	NC	y		y	y	++	ECRC
	TW04272	H	y		y	y	−	*49*
	99.0703	B	y		y	y	+	ECRC
	P5-1	B	y		y	y	++	*23*
O111:H8	1056-1	B	y	y		y	+	MARC
	DECA 8B	H	y	y	y	y	−	*49*
	BEO0-749	H	y		y	y	+	TPH
	7-14 10A	B	y		y	y	++	MARC
	DEC 8A	H	y		y	y	−	NFSTC
	90.0102	B	y	y	y	y	−	ECRC
	IDPH14614	H	y	y	y	y	−	IDHW
	7-16 64A	F	y	y	y	y	+	MARC
	7-45 80A	F	y		y	y	++	MARC
	TW05614	H	y	y	y	y	−	*49*

[a] G, ground beef; H, human; B, bovine; F, fly; NC, not confirmed.
[b] Emply cells, not detected; y, yes.
[c] Curli expression was confirmed by streaking the strains onto Congo red indicator (CRI) agar plates. Curli expression level was evaluated based on the pigmentation of colonies on CRI plates. −, negative; ±, weak positive; +, positive; ++, strong positive.
[d] MH, provided by Dr. Marcus Head, USDA, FSIS; ES, provided by Evangaline Sowers, DHHS, CDC, NCID; IDHW, provided by Idaho Department of Health and Welfare; NFSTC, National Food Safety and Toxicology Center (available at: http://www.shigatox.net/cgi-bin/deca); MARC, U.S. Meat Animal Research Center culture collection; ECRC, *Escherichia coli* Reference Center (available at: http://ecoli.cas.psu.edu/); TPH, provided by the Texas Department of Health.

phosphate-buffered saline (PBS; pH 7.2) to remove loosely attached cells and then air dried and stained with 100 μl per well of 0.1% CV for 20 min at 22 to 25°C. The plates were washed again with PBS and air dried, and the remaining CV was dissolved in 100 μl per well of 85% ethanol. The amount of the extracted CV in each well was determined by measuring A_{570} using the microplate reader. Each data point was averaged from at least six replicate wells. The experiments were performed five times using independent cultures.

Pellicle formation at air-liquid interfaces in glass tubes. To compare the ability of various STEC strains to form pellicles at the air-liquid interface, overnight bacterial cultures were diluted 1:100 in fresh LB-LS broth and added to glass tubes (16 by 150 mm; Kimble Glass Inc., Eugene, OR) at 2 ml per tube. Tubes of sterile LB-LS broth were used as negative control samples, and all tubes were incubated statically for 5 days at 22 to 25°C. Supernatants were gently removed at the end of the incubation period, and all tubes were washed with 3 ml per tube of PBS. The tubes were air dried, stained with 3 ml per tube of 0.1% CV for 20 min at 22 to 25°C, and then washed twice with 3 ml per tube of PBS. The tubes were air dried again, and pellicle formation at the air-liquid interfaces was photographed using a digital camera for visual comparison. The remaining CV in each tube was dissolved in 4 ml of 85% ethanol by vortexing and was transferred to a new 96-well plate at 200 μl per well for optical density at 570 nm (OD_{570}) measurement using the microplate reader. Each data set was the average of results from at least three replicate wells. The experiments were repeated three times using independent cultures.

Tolerance of STEC biofilm cells to sanitizing agents. The two types of sanitizers described above were applied to test the survival capability of different STEC cells in biofilms. Three STEC strains that exhibited curli expressions at different levels and showed either high or moderate potency of biofilm formation in the previous tests were selected from each serotype and allowed to form biofilms on 96-well polystyrene plates for 72 h as described above. At the end of the incubation period, bacterial supernatants were gently removed by aspiration, and the plates were washed with 200 μl per well of sterile PBS to remove the planktonic cells or any loosely attached cells. The plates were air dried for 5 min at 22 to 25°C, and then the wells were filled with 200 μl of sterile PBS, a 300-ppm QAC solution, or 200 ppm of chlorine. All

TABLE 2. *Quantitative comparison of biofilm formation by STEC strains of serotypes O157:H7, O26:H11, and O111:H8 on 96-well polystyrene plates*[a]

Serotype	Strain	Incubation time (h): 24	48	72
O157:H7	FSIS 62	−0.143 ± 0.07	0.323 ± 0.09	**1.813 ± 0.23**
	TX 909-1	0.120 ± 0.09	0.310 ± 0.07	**1.443 ± 0.20**
	USDA 39	0.091 ± 0.04	**0.433 ± 0.10**	**0.939 ± 0.07**
	FSIS 71	−0.180 ± 0.04	0.262 ± 0.08	**0.743 ± 0.11**
	FSIS 53	0.106 ± 0.08	0.339 ± 0.04	**0.606 ± 0.13**
	FSIS 78	−0.117 ± 0.05	0.229 ± 0.06	**0.506 ± 0.09**
	USDA 2	0.105 ± 0.15	0.331 ± 0.05	**0.418 ± 0.11**
	RM 6049	−0.014 ± 0.05	0.268 ± 0.05	**0.390 ± 0.09**
	FSIS 11	−0.032 ± 0.03	**0.388 ± 0.08**	**0.360 ± 0.07**
	FSIS 10	0.090 ± 0.06	0.312 ± 0.11	0.244 ± 0.05
O26:H11	7-14 50A	−0.071 ± 0.04	0.296 ± 0.05	**1.720 ± 0.29**
	P5-1	**0.428 ± 0.15**	**1.022 ± 0.20**	**1.154 ± 0.24**
	DECA 10A	−0.151 ± 0.05	0.185 ± 0.03	**0.997 ± 0.12**
	10205	0.118 ± 0.06	0.215 ± 0.04	**0.795 ± 0.08**
	CL5	−0.078 ± 0.05	**0.721 ± 0.24**	**0.595 ± 0.12**
	DEC 10E	0.063 ± 0.07	0.243 ± 0.06	**0.573 ± 0.11**
	DEC 10B	−0.063 ± 0.10	0.285 ± 0.07	**0.534 ± 0.11**
	18 B24-2	−0.012 ± 0.12	0.313 ± 0.04	**0.380 ± 0.09**
	99.0703	0.093 ± 0.04	0.198 ± 0.04	0.227 ± 0.07
	TW04272	−0.111 ± 0.04	0.269 ± 0.06	0.122 ± 0.05
O111:H8	TW05614	**1.415 ± 0.24**	**2.782 ± 0.16**	**2.576 ± 0.15**
	7-14 10A	−0.067 ± 0.04	0.188 ± 0.06	**1.332 ± 0.32**
	1056-1	−0.019 ± 0.05	0.267 ± 0.04	**0.953 ± 0.34**
	7-45 80A	0.162 ± 0.14	**1.382 ± 0.18**	**0.707 ± 0.15**
	BEO0-749	−0.013 ± 0.09	0.095 ± 0.02	**0.654 ± 0.12**
	DECA 8B	−0.055 ± 0.07	0.279 ± 0.07	**0.620 ± 0.16**
	7-16 64A	0.197 ± 0.13	**0.701 ± 0.13**	**0.555 ± 0.12**
	90.0102	0.144 ± 0.07	**0.633 ± 0.11**	**0.402 ± 0.11**
	IDPH14614	0.113 ± 0.09	**0.817 ± 0.16**	0.300 ± 0.06
	DEC 8A	0.083 ± 0.08	**0.393 ± 0.11**	0.295 ± 0.05

[a] Bacterial strains were incubated statically in LB-LS broth for 24, 48, or 72 h at room temperature, and biofilm formation was quantified with crystal violet staining as described in ''Materials and Methods.'' Six microtiter plate wells were used for each sample. Data are shown as mean adjusted OD ± standard error. Mean adjusted OD represents the difference between the OD (A_{570}) of the samples and the OD of the negative controls. The LSD ($P < 0.05$) among means was 0.350. Samples with mean adjusted OD > 0.350 (indicated by boldface) had significant biofilm formation ($P < 0.05$).

sanitizing solutions were removed by aspiration after incubation at 22 to 25°C for 2 or 10 min. Then the samples were neutralized by adding 170 μl per well of Dey/Engley broth (BBL, Difco, Sparks, MD) supplemented with 0.3% soytone and 0.25% sodium chloride, and the remaining cells in each well were harvested by scraping the surface with sterile pipette tips and rinsing the well with the neutralizing broth. Bacterial cells harvested from six replicate wells that received the same sanitization treatment were combined into ~1 ml of neutralizing broth, vigorously vortexed to disrupt cell aggregates, and then serially diluted in fresh neutralizing broth and plated onto PetriFilm AC plates (3M Microbiology, St. Paul, MN). The plates were incubated overnight (16 to 20 h) at 37°C, and then colonies on the plates were counted to determine the number of STEC cells present.

Statistical analysis. Adjusted OD (A_{570}) was calculated as the difference between the OD of the samples and the OD of the negative controls. Data were analyzed with the generalized linear model procedure of SAS with fixed effects of species and strain within species, which was tested against the species sums of squares. When the OD data of biofilm formation on 96-well plates were analyzed, the model also included incubation time and the interaction of strains (species) and incubation time. The least significant difference (LSD; $P < 0.05$) was calculated using the probability of difference option. Data of bacterial colony counts from sanitization experiments were log transformed and analyzed with the generalized linear model procedure of SAS with fixed effects of sanitizer, exposure time, species, and strain within species, which was tested against the species sums of squares. All possible interactions were tested, and the LSD ($P < 0.05$) among means for the three-way interaction was calculated using the probability of difference option.

RESULTS

Detection and quantification of STEC biofilm formation on polystyrene microtiter plates. Biofilm formation on 96-well polystyrene plates was measured with CV staining as an initial phenotype screening to compare the biofilm-forming ability by various STEC strains of serotype O157:H7, O26:H11, or O111:H8. Ten strains of each serotype were used to determine biofilm formation after incubation at room

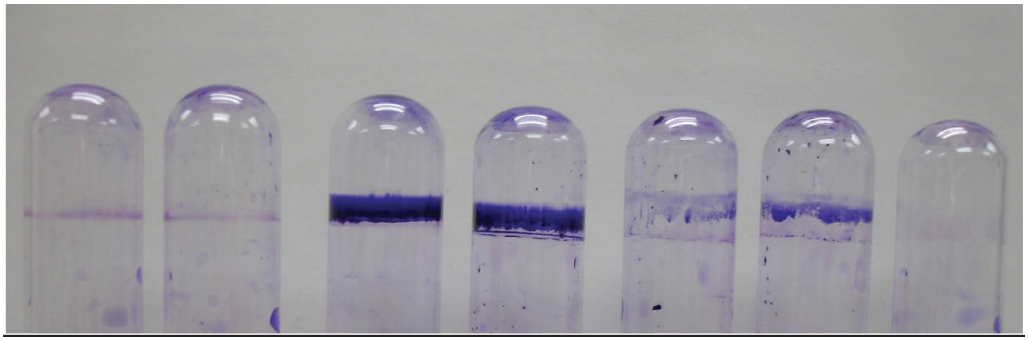

FIGURE 1. *Typical pellicle formation by STEC strains of serotypes O157:H7, O26:H11, and O111:H8 at air-liquid interfaces in glass tubes. Bacterial strains were incubated statically in LB-LS broth for 5 days at room temperature, and pellicle formation was visualized with crystal violet staining as described in ''Materials and Methods.'' From left to right: STEC O157:H7 strains FSIS 71 and FSIS 62; STEC O26:H11 strains 10205 and 7-14 50A; STEC O111:H8 strains 1056-1 and 7-14 10A; the negative control tube with sterile LB-LS broth only.*

temperature for 24, 48, or 72 h. A_{570} showed that biofilm formation was generally enhanced with increasing incubation time, as the majority of the strains developed the highest biofilm mass at 72 h postinoculation (Table 2). Based on statistical analysis of the data, only two strains, O26:H11 strain P5-1 and O111:H8 strain TW05614, developed significant biofilms compared with negative controls after the first 24 h of incubation. A plateau of biofilm development was also observed in several strains by 48 h of incubation, or a slight decrease of OD_{570} values by 72 h of incubation, mostly in the O111:H8 strains.

Within serotype O157:H7, strain FSIS 62 had the highest mean $\pm$ standard error (SE) OD_{570} value (1.813 $\pm$ 0.23) by 72 h, followed by strain TX 909-1 (1.443 $\pm$ 0.20) and strain USDA 39 (0.939 $\pm$ 0.07). OD_{570} values of strain FSIS 10 showed no significant difference from medium controls at all time points. Among the O26:H11 strains, strain 7-14 50A demonstrated the strongest biofilm formation, with an OD_{570} value of 1.720 $\pm$ 0.29 after 72 h of incubation, followed by strains P5-1 (1.154 $\pm$ 0.24), DECA 10A (0.997 $\pm$ 0.12), and 10205 (0.795 $\pm$ 0.08). Strains 99.0703 and TW04272 of serotype O26:H11 did not form significant biofilms at all testing time points. For serotype O111:H8, strain TW05614 formed the highest biofilm mass among the 10 O111:H8 strains, with OD_{570} values of 2.782 $\pm$ 0.16 and 2.576 $\pm$ 0.15 by 48 and 72 h, respectively, followed by strain 7-14 10A (1.332 $\pm$ 0.32) and strain 1056-1 (0.953 $\pm$ 0.34), forming the most biofilm mass after 72 h of incubation. Strains 7-45 80A, 7-16 64A, IDPH 14614, 90.0102, and DEC 8A each formed biofilms significantly higher than medium controls by 48 h, but showed a decrease of biofilm mass at various levels after 72 h of incubation. Notably, statistical analysis of variance (ANOVA) showed significant influences of both strain and incubation time on biofilm development; however, no significant influence of the serotypes was observed. These data suggested that STEC biofilm formation on polystyrene surfaces was highly dependent on the strains but not the serotypes, since no particular serotype among the three showed higher potency compared with the others. Other experimental conditions, such as incubation time period, also played an important role in biofilm development in this experimental setting.

Pellicle formation at the air-liquid interface on glass surfaces. Another feature characteristic of biofilm formation, the formation of a pellicle at the air-liquid interface, was examined in glass tubes using the same strains. When grown statically at room temperature for 5 days, all O157:H7 strains showed pellicle formation at low levels. However, under the same conditions, O26:H11 strains 7-14 50A and 10205 each formed a very dense pellicle by the edge of the liquid culture at the interface, followed by strains 18 B24-2, DEC 10E, and 99.0703. Meanwhile, O111:H8 strains 7-14 10A, TW05614, 7-45 80A, 7-16 64A, BEOO-749, and 1056-1 all demonstrated significant pellicle formation compared with the negative control tubes containing only the sterile LB broth (Fig. 1 and Table 3). When the pellicle in each tube was stained with CV and dissolved in 85% ethanol, and the OD_{570} was measured, statistical ANOVA showed significant influences of both the strain and the serotype on pellicle formation, indicating that serotype O26:H11 had the highest potency of pellicle formation at air-liquid interface on glass surfaces, followed by serotype O111:H8. Overall, serotype O157:H7 exhibited limited to no capacity for pellicle formation in this experimental setting.

Bacterial survival in sanitizer-treated biofilms. Table 4 represents the mean of total surviving bacteria (log CFU per well) in biofilms formed by different strains after being treated with PBS, a 300-ppm QAC solution, or 200 ppm of chlorine. The number of viable cells recovered from PBS-treated biofilms was used as a positive control for each strain in order to compare their surviving capability after sanitizer treatment for the different time periods. After removing planktonic cells and loosely attached bacteria with PBS washing, the cell density in biofilms formed on 96-well polystyrene plates reached approximately 7.0 to 8.0 log CFU per well. Notably, for each individual strain, similar cell densities were recovered from biofilms washed by PBS for 2 or 10 min. However, for each individual strain, significant cell reductions were achieved by each sanitizer with either 2- or 10-min exposure time, calculated by statistical analysis of means with the LSD among means at 0.845 ($P < 0.05$).

QAC solution. Compared with PBS washing, bacterial survival after the 300-ppm QAC treatment was highly

TABLE 3. *Quantitative comparison of pellicle formation by STEC strains of serotypes O157:H7, O26:H11, and O111:H8 at air-liquid interfaces in glass tubes*[a]

Serotype	Strain	OD_{570}
O157:H7	FSIS 71	**0.320 ± 0.02**
	USDA 2	0.251 ± 0.03
	FSIS 10	0.235 ± 0.02
	TX 909-1	0.218 ± 0.03
	FSIS 53	0.204 ± 0.03
	FSIS 78	0.152 ± 0.03
	USDA 39	0.146 ± 0.02
	RM 6049	0.144 ± 0.01
	FSIS 11	0.118 ± 0.01
	FSIS 62	0.060 ± 0.04
O26:H11	10205	**3.187 ± 0.03**
	7-14 50A	**3.064 ± 0.17**
	DEC 10E	**2.138 ± 0.02**
	18 B24-2	**2.072 ± 0.06**
	99.0703	**1.428 ± 0.03**
	TW04272	**0.531 ± 0.02**
	DEC 10B	**0.457 ± 0.02**
	DECA 10A	**0.437 ± 0.03**
	CL5	**0.398 ± 0.02**
	P5-1	**0.357 ± 0.02**
O111:H8	7-14 10A	**1.351 ± 0.10**
	TW05614	**1.343 ± 0.13**
	7-45 80A	**1.234 ± 0.05**
	BEO0-749	**0.983 ± 0.07**
	7-16 64A	**0.947 ± 0.03**
	1056-1	**0.791 ± 0.03**
	IDPH 14614	**0.352 ± 0.03**
	90.0102	**0.330 ± 0.03**
	DECA 8B	0.251 ± 0.01
	DEC 8A	0.225 ± 0.03

[a] Bacterial strains were incubated statically in LB-LS broth for 5 days in glass tubes at room temperature, and pellicle formation was quantified with crystal violet staining as described in ''Materials and Methods.'' Data are shown as mean adjusted OD ± standard error. Mean adjusted OD represents the difference between the OD (A_{570}) of the samples and the OD of the negative controls. The LSD ($P < 0.05$) among means was 0.260. Samples with mean adjusted OD > 0.260 (indicated by boldface) had significant pellicle production ($P < 0.05$).

strain-dependent. QAC exposure for 2 min resulted in an approximately 2.0 log CFU per well reduction of STEC O157:H7 biofilm cells. Prolonged periods of QAC treatment led to further reductions of viable cell populations. A 10-min QAC exposure reduced viable cells of strain FSIS 62 from 7.47 to 4.37 log CFU per well, a 3.0-log CFU per well reduction. For the two curli-negative O157:H7 strains, TX 909-1 and USDA 39, the longer period of QAC exposure reduced biofilm cells from 7.35 to 1.47 and from 7.40 to 1.02 log CFU per well, respectively. When the O26:H11 biofilms were treated with the 300-ppm QAC solution, a 2-min exposure led to 1.67- and 2.88-log CFU per well reductions of biofilm cells by the curli-positive strains 7-14 50A and 10205, respectively. A 10-min treatment reduced these biofilm cells by 2.79 and 2.71 log CFU per well. However, viable cells in biofilms formed by strain DECA 10A, an O26:H11 strain with weak curli expression, were reduced to a negligible level of 0.76 log CFU per well with either 2- or 10-min QAC exposure. Similar results were also obtained when the O111:H8 biofilms were treated with QAC solution. Viable cells in biofilms formed by curli-negative O111:H8 strain TW05614 were reduced to 1.28 and 1.55 log CFU per well by 2- and 10-min QAC exposures, respectively, each representing an over 6.0-log CFU per well reduction. However, biofilms formed by the curli-positive O111:H8 strains 7-14 10A and 1056-1 exhibited higher tolerance to the treatment. Cell densities of these biofilms were reduced by approximately 1.0 and 2.0 log CFU per well following 2- and 10-min QAC exposures, respectively.

Chlorine. The STEC biofilms exhibited tolerance to chlorine treatment at different levels, which was also highly strain-dependent. Exposure to 200 ppm of chlorine for 2 or 10 min each reduced biofilm cell densities of curli-positive O157:H7 strain FSIS 62 by approximately 3.0 log CFU per well. For the curli-negative O157:H7 strains, 2- and 10-min chlorine treatments reduced viable cells of strain TX 909-1 by 1.97 and 3.66 log CFU per well, respectively. Chlorine treatment also reduced viable cells in biofilms formed by curli-negative strain USDA 39 to 3.16 log CFU per well (a 4.27-log reduction) and to 0.71 log CFU per well (a 6.69-log reduction) after 2- and 10-min exposure, respectively. This treatment reduced viable cell counts in biofilms formed by O26:H11 strain DECA 10A with a weak curli expression to 2.95 log CFU per well (a 4.18-log reduction) after a 2-min exposure, and to a negligible level of 0.18 log CFU per well (a 6.91-log reduction) after a 10-min exposure. Biofilms formed by the two curli-positive O26:H11 strains were less sensitive to chlorine treatment. Exposure of these biofilms to 200 ppm of chlorine resulted in approximately 2.0- to 3.0-log cell reductions after a 2-min exposure, and 3.0- to 4.0-log cell reductions with a 10-min exposure. For the O111:H8 strains, 200 ppm of chlorine treatments for either 2 or 10 min were effective in deactivating biofilms formed by curli-negative strain TW05614, both reducing viable cell counts to lower than 1.0 log CFU per well. However, biofilms formed by the two curli-positive O111:H8 strains were much more tolerant to the same treatments, which only reduced viable bacterial counts by 1.0 to 2.0 log CFU per well for strains 7-14 10A and 1056-1.

Thus, of the STEC strains that we tested, the curli-negative strains were overall more sensitive to both types of sanitizers, while biofilms formed by the curli-positive STEC strains were more tolerant. However, statistical analysis of means indicated that there was no significant difference in cell reductions among the three STEC serotypes with either sanitizer treatment.

DISCUSSION

Biofilm formation is one of the major strategies that support bacterial survival under adverse circumstances. Bacteria can form biofilms on a wide variety of solid surfaces and at the air-liquid or liquid-solid interfaces. It has been shown that STEC may form biofilms in different areas

TABLE 4. *Bacterial survival in STEC biofilms formed on 96-well polystyrene plates after treatments with PBS, QAC, or chlorine solutions*[a]

Serotype	Strain	Curli expression[b]	Sanitization treatment					
			2-min exposure			10-min exposure		
			PBS	QAC	Chlorine	PBS	QAC	Chlorine
O157:H7	TX 909-1	−	7.28 (0.18)	5.89 (0.31)	5.31 (0.12)	7.35 (0.27)	1.47 (1.62)	3.69 (0.59)
	USDA 39	−	7.43 (0.19)	4.78 (0.15)	3.16 (0.32)	7.40 (0.20)	1.02 (1.12)	0.71 (0.78)
	FSIS 62	±	7.50 (0.24)	5.43 (0.06)	4.63 (0.24)	7.47 (0.14)	4.37 (0.08)	4.42 (0.13)
O26:H11	7-14 50A	++	7.47 (0.37)	5.80 (0.05)	5.00 (1.05)	7.32 (0.26)	4.53 (1.16)	3.98 (1.83)
	DECA 10A	±	7.13 (0.22)	0.76 (0.83)	2.95 (1.30)	7.09 (0.06)	0.76 (0.83)	0.18 (0.31)
	10205	+	7.34 (0.21)	4.46 (0.57)	3.92 (0.76)	7.32 (0.16)	4.61 (0.65)	2.79 (1.95)
O111:H8	TW05614	−	7.88 (0.49)	1.28 (1.40)	0.20 (0.49)	7.91 (0.15)	1.55 (1.69)	0.96 (0.12)
	7-14 10A	++	8.42 (0.15)	7.25 (0.13)	6.26 (0.91)	8.11 (0.12)	5.73 (0.52)	5.97 (0.93)
	1056-1	+	8.08 (0.17)	7.00 (0.07)	7.15 (0.07)	7.91 (0.08)	6.00 (0.69)	5.72 (1.43)

[a] QAC and chlorine solutions were prepared in sterile distilled water and used at final concentrations of 300 ppm and 200 ppm, respectively. Data are shown as mean log CFU per well (standard error) ($n = 12$). All possible interactions of sanitizer, exposure time, species, and strain within species were analyzed, and the LSD ($P < 0.05$) among means was 0.845.

[b] −, negative; ±, weak positive, ++, strong positive; +, positive.

of food processing environments, such as floors, walls, pipes, and drains, etc. *(25, 27)*. A wide variety of materials commonly used for food processing equipment may also become hosts for STEC biofilms, such as stainless steel, aluminum, nylon, Teflon, rubber, plastic, glass, and polyurethane, etc. *(39)*. Because detached biofilms may serve as a continuous contamination source and biofilm cells are usually more tolerant to sanitization than their planktonic counterparts, it is important to investigate biofilm formation by various STEC serotypes and biofilm tolerance to sanitizers in order to provide information regarding properly sanitizing food contact surfaces with the most effective reagents.

In the present study, we first compared the potential of biofilm formation by various STEC strains of serotypes O157:H7, O26:H11, and O111:H8. Biofilm formation on polystyrene surfaces appeared to be a strain-dependent phenomenon regardless of the serotype (Table 2). Statistical ANOVA indicated that only the factors of strain and incubation time affected biofilm development in this setting. This is consistent with previous studies conducted by Dourou et al. *(10)* using a collection of O157:H7 strains. They found that bacterial attachment to beef fabrication surfaces was highly strain-dependent, suggesting that strain selection was important for STEC biofilm research *(10)*. Similar results were also obtained from a more recent study *(2)* that determined the presence of adhesion-related genes and compared biofilm-forming ability in 18 O157:H7 strains and 33 non-O157 strains belonging to serotypes O26, O111, O103, and O145. This study concluded that, under defined culture conditions, the ability to develop biofilms on abiotic surfaces (96-well plates) varied from strain to strain but was not restricted to any particular serotype.

However, we obtained different observations when testing pellicle formation at the air-liquid interfaces on glass surfaces. We found that all O157:H7 strains exhibited weak pellicle-forming ability, whereas five O26:H11 strains and six O111:H8 strains formed dense pellicles (Fig. 1 and Table 3). Statistical analysis indicated that factors of both strain and serotype played significant roles in this phenomenon. It has been known that STEC biofilm formation could be influenced by a variety of factors, including the characteristics of the strains, physical and chemical properties of the surface materials for cell attachment, bacterial growth phases, growth medium, and other environmental conditions. In our settings, the different surface materials for cell attachment and the sufficient air exposure available at the air-liquid interfaces may have played a role in the differences that we observed. Numerous studies have shown that surface materials had significant effects on bacterial adhesion and biofilm formation *(25, 50)*. STEC strains also demonstrated different biofilm-forming potential on hard surfaces made of various materials. In particular, the O157:H7 strains were found to form biofilms most efficiently on stainless steel and on high density polyethylene surfaces; and the O157:H7 biofilms on stainless steel were able to transfer the bacteria to meat, poultry, and other food products *(39, 40)*.

Biofilm formation has also been shown to be mediated by the combination of bacterial surface structures expressed at various levels dependent upon the environmental conditions encountered by the bacteria. Among those, the expression of Congo red–binding curli fimbriae and exopolysaccharide cellulose has been well associated with STEC biofilm formation. It was reported that 46 to 79% of the commensal *E. coli* isolates express either one or both components, and these strains often demonstrated higher biofilm-forming capabilities *(44)*. Previous studies *(43)* indicated that the majority of O157:H7 strains did not express curli fibers; therefore, these strains were generally not strong biofilm formers in the laboratory. Furthermore, one study *(26)*, which detected biofilms formed by seven O157:H7 strains on stainless steel and polyurethane coupons with direct epifluorescence microscopy, observed that these biofilms were all composed of small microcolonies and single cells, most likely due to the lack of curli

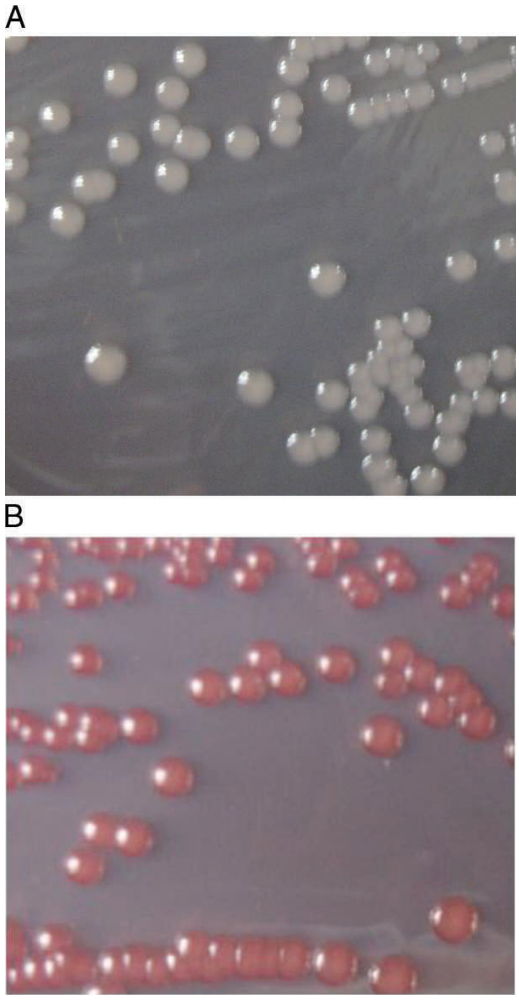

FIGURE 2. *Curli expression of STEC strains on Congo red indicator agar plates. Overnight cultures of STEC O157:H7 strain FSIS10 (A) and O26:H11 strain 7-14 50A (B) were streaked onto Congo red indicator agar plates, and the plates were incubated at 25°C for 48 h.*

expression. A study by Cookson et al. *(9)* investigated the role of type 1 and curli fimbriae in STEC biofilm formation using 13 O157:H7 strains and 13 non-O157 strains belonging to serotypes O26, O128, O103, and O145. All 13 O157:H7 strains showed negative fimbrial expression, but 11 of 13 and 5 of 13 non-O157 strains expressed type 1 fimbriae and curli fimbriae, respectively. Their data further indicated that elaboration of curli fimbriae enhanced STEC biofilm formation. In the present study, we also screened all 30 STEC strains for curli expression on CRI plates (Fig. 2), and our observations were in agreement with the previous reports (Table 1). Of the 10 O157:H7 strains, FSIS 62 was the only strain that exhibited a weak curli expression on CRI plates. Notably, this strain also displayed the highest potency of biofilm formation on polystyrene plates among the 10 O157:H7 strains. In contrast, 7 of the 10 O26:H11 strains and 5 of the 10 O111:H8 strains demonstrated positive curli expression at various levels, and a correlation between positive curli expression and strong biofilm formation on polystyrene plates was also observed in most of these strains. These data were consistent with results of previous studies showing that curli expression by *E. coli* strains could enhance cell attachment on polystyrene surfaces *(35, 46)*. In tests at the air-liquid interface on glass surfaces, the curli-positive strains of serotype O111:H8 also exhibited an overall high potency of pellicle formation. However, not all curli-positive strains of serotype O26:H11 formed strong pellicles in the same setting, for example, strains CL-5 and P5-1. On the other hand, two O26:H11 strains, 18 B24-2 and DEC 10E, displayed negative curli expression on CRI plates and formed very weak biofilms on polystyrene plates but demonstrated strong pellicle-forming capability (Table 3). In addition, a curli-negative O111:H8 strain, TW05614, showed a high potential of biofilm formation on polystyrene surfaces (Table 2). These observations suggest that other genetic determinants are involved in the phenomenon, as the genes required for biofilm or pellicle formation by the various serotypes and strains might differ. Indeed, the regulation of the bacterial quorum sensing system, chemotaxis, flagellar synthesis, and other motility genes was found to be involved in STEC biofilm formation at various levels under different conditions *(39)*. Therefore, selections of bacterial strains and experimental conditions should be examined on a case-by-case basis since STEC biofilm formation is affected in complex ways by a combination of physiological and environmental factors.

Virulent STEC strains can cause severe human illness with a low infectious dose; therefore, these foodborne pathogens present a serious health risk through cross-contamination from food contact surfaces to food products. The strong potential of biofilm formation by certain STEC strains and their increased tolerance to environmental stresses highlights the importance of properly sanitizing food processing equipment and food contact surfaces in order to control and inactivate STEC biofilms. A 3-log reduction (99.9%) of viable cells has been considered as an effective decontamination of attached or biofilm-forming bacteria *(30)*. However, an area not completely explored is the tolerance of biofilms formed by various STEC serotypes to the common sanitizers, especially the non-O157 STEC biofilms. The effectiveness of sanitizers against STEC biofilms could be influenced by a combination of factors such as the materials of the attachment surfaces, the bacterial strains, and the age of the biofilms. In general, mature biofilms are more tolerant to physical and chemical treatment due to the strong three-dimensional structures and the additional protections provided by the multiple layers of bacterial cells with well-expressed extracellular substances. To test biofilm tolerance to sanitizers, from each STEC serotype we selected three strains that demonstrated high or moderate potential of biofilm formation on polystyrene plates; we let them form biofilms for 72 h as most of these strains developed the highest biofilm mass after 72 h of incubation in the previous experiments.

Two types of sanitizers commonly used in the food industry were tested for their effectiveness against the STEC biofilms. Vanquish is a QAC-based, one-step sanitizer effective against bacteria, viruses, and fungi. Its bactericidal activity is associated with the C_{12}–C_{14} alkyl derivatives in the active ingredients that can disrupt cell

membrane lipid bilayers, compromise cellular permeability controls, and induce leakage of cellular contents. This sanitizer is commonly applied in federally inspected meat and poultry plants, and it can be used on a wide variety of food contact surfaces without requiring a rinse. Chlorine-based sanitizers, available in solid, liquid, or gas-injection forms, are the most commonly used reagents in food processing plants. Chlorine sanitizers act as oxidizing agents on thiol groups, and they are generally effective against a wide variety of bacteria. The use of chlorine has been evaluated and suggested as an effective means to depolymerize and remove the exopolysaccharide matrix that surrounds and protects bacterial biofilms against sanitization *(24).* Usually no water rinse is required if chlorine concentration in the solution is lower than 200 ppm. Overall, our findings indicated that significant reductions of viable biofilm cells were achieved with each sanitizer, and STEC biofilm tolerance to sanitization was highly strain-dependent regardless of the serotype. Recent studies conducted at our institute indicated that, on pathogen-inoculated fresh beef surfaces, the non-O157 STEC cells were as sensitive as the O157:H7 cells to antimicrobial interventions using lactic acid, acidified sodium chlorite, or peroxyacetic acid (unpublished data). This was in agreement with our present sanitization study targeting STEC biofilms formed on solid surfaces, as we observed no significant difference in cell reductions among the three STEC serotypes.

As described above, curli expression was correlated with enhanced STEC biofilm formation. Moreover, Ryu et al. *(37)* previously reported that curli production significantly increased the resistance of STEC O157:H7 biofilms to chlorine exposure. In addition, a recent study presented genetic evidence indicating a contribution of curli expression to *E. coli* cell survival in the presence of toxic compounds, such as nickel *(17).* In our sanitization study we also observed that curli expression by O157:H7, and the non-O157 STEC strains was associated with their high tolerance to sanitizer treatments (Table 4). Among the O157:H7 strains, the average decrease in viable cells of the two curli-negative strains was approximately 6.0 log CFU per well, but that of O157:H7 strain FSIS 62 with a weak curli expression was 3.0 log CFU per well after a 10-min QAC exposure. For the non-O157 strains with negative or low curli expressions (O26:H11 strain DECA 10A and O111:H8 strain TW05614), QAC exposure for 2 or 10 min both reduced biofilm cells to negligible levels of approximately 1.0 log CFU per well, which represents more than 6.0-log reductions in each case. However, QAC treatment for 2 or 10 min only reduced viable cells of the non-O157 strains with strong curli expressions by an average of 1.0 to 3.0 log CFU per well. A similar pattern was also observed with chlorine treatment. The average cell reduction of the curli-negative STEC strains was more than that of the curli-positive strains following a 2-min chlorine exposure. When the contact time was increased to 10 min, viable cells of the three STEC stains with low or negative curli expressions were further reduced to negligible levels of lower than 1.0 log CFU per well. Meanwhile, the curli-positive stains exhibited higher tolerance, as the 10-min chlorine exposure resulted in an additional 1.0-log cell reduction for O26:H11 strain 7-14 50A and O111:H8 strain 1056-1, and no significant difference in cell reduction was observed between 2- and 10-min chlorine treatments for O157:H7 stain FSIS 62 and O26:H11 strain 10205. The two curli-positive O111:H8 strains demonstrated the highest tolerance to both sanitizers. The average decreases in viable cells for these two strains were 1.0 and 2.0 log CFU per well, following 2- and 10-min exposure to either sanitizer, respectively. These observations suggested that curli expression by STEC strains was involved in not only enhancing biofilm formation but also in protecting the biofilms against sanitizing procedures. Notably, among the three O111:H8 strains, the curli-negative strain TW05614 demonstrated the highest potency of biofilm formation on polystyrene plates but the lowest tolerance to both sanitizers, suggesting that a high potential of cell attachment and biofilm mass development does not necessarily parallel a high tolerance to sanitizers. Different gene regulations could be initiated in response to sanitization and to other adverse stress that triggers cell adhesion and biofilm formation.

In conclusion, our study showed that biofilm formation by STEC strains of serotypes O157:H7, O26:H11, and O111:H8 was highly strain-dependent on polystyrene surfaces, whereas the two non-O157 serotypes demonstrated a higher potency of pellicle formation at air-liquid interfaces on glass surfaces. This phenomenon might be affected by a combination of bacterial physiological as well as environmental factors. Significant reductions of viable biofilm cells were achieved after treatments with sanitizers commonly used in the food industry, including chlorine and a QAC solution. No significant difference was observed in cell reductions by sanitization between the non-O157 and O157:H7 biofilms. However, biofilms formed by curli-positive STEC strains exhibited higher tolerance to the sanitizing procedures; therefore, bacterial curli expression played a significant role not only in STEC biofilm formation but also in high tolerance to sanitizers as well. The strong potency of biofilm and pellicle formation by various STEC serotypes and their high tolerance to sanitization might contribute to STEC colonization on food contact surfaces and the resultant cross-contamination, which could lead to foodborne outbreaks and clinical infections.

ACKNOWLEDGMENTS

The authors thank Sydney Brodrick, Bruce Jasch, and Frank Reno for technical support, and Muerilyn Bierman for secretarial assistance.

REFERENCES

1. Bielaszewska, M., W. Zhan, A. Mellmann, and H. Karch. 2007. Enterohaemorrhagic *Escherichia coli* O26:H11/H-: a human pathogen in emergence. *Berl. Muench. Tieraerztl. Wochenschr.* 120:279–287.
2. Biscola, F. T., C. M. Abe, and B. E. Guth. 2011. Determination of adhesion gene sequences and biofilm formation in O157 and non-O157 Shiga toxin–producing *Escherichia coli* strains isolated from different sources. *Appl. Environ. Microbiol.* 77:2201–2208.
3. Bono, J. L., J. E. Keen, M. L. Clawson, L. M. Durso, M. P. Heaton, and W. W. Laegreid. 2007. Association of *Escherichia coli* O157:H7 *tir* polymorphisms with human infection. *BMC Infect. Dis.* 7:98.

4. Bono, J. L., J. E. Keen, L. C. Miller, J. M. Fox, C. G. Chitko-McKown, M. P. Heaton, and W. W. Laegreid. 2004. Evaluation of a real-time PCR kit for detecting *Escherichia coli* O157 in bovine fecal samples. *Appl. Environ. Microbiol.* 70:1855–1857.
5. Brooks, J. T., E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra, and N. A. Strockbine. 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983–2002. *J. Infect. Dis.* 192:1422–1429.
6. Calderon, V. E., Q. Chang, M. McDermott, M. B. Lytle, G. McKee, K. Rodriguez, D. A. Rasko, V. Sperandio, and A. G. Torres. 2010. Outbreak caused by cad-negative Shiga toxin-producing *Escherichia coli* O111, Oklahoma. *Foodborne Pathog. Dis.* 7:107–109.
7. Carter, M. Q., M. T. Brandl, J. W. Louie, J. L. Kyle, D. K. Carychao, M. B. Cooley, C. T. Parker, A. H. Bates, and R. E. Mandrell. 2011. Distinct acid resistance and survival fitness displayed by Curli variants of enterohemorrhagic *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 77:3685–3695.
8. Clawson, M. L., J. E. Keen, T. P. Smith, L. M. Durso, T. G. McDaneld, R. E. Mandrell, M. A. Davis, and J. L. Bono. 2009. Phylogenetic classification of *Escherichia coli* O157:H7 strains of human and bovine origin using a novel set of nucleotide polymorphisms. *Genome Biol.* 10:R56.
9. Cookson, A. L., W. A. Cooley, and M. J. Woodward. 2002. The role of type 1 and curli fimbriae of Shiga toxin-producing *Escherichia coli* in adherence to abiotic surfaces. *Int. J. Med. Microbiol.* 292:195–205.
10. Dourou, D., C. A. Simpson, K. E. Belk, J. A. Scanga, G. C. Smith, and K. Koutsoumanis. 2008. Variation on the attachment and biofilm formation among *Escherichia coli* O157:H7 strains on stainless steel surfaces under two inoculation scenarios, 010-28. Abstr. 68th Annu. Meet. Inst. Food Technol., New Orleans, LA.
11. Durso, L. M., J. L. Bono, and J. E. Keen. 2005. Molecular serotyping of *Escherichia coli* O26:H11. *Appl. Environ. Microbiol.* 71:4941–4944.
12. Durso, L. M., J. L. Bono, and J. E. Keen. 2007. Molecular serotyping of *Escherichia coli* O111:H8. *J. Microbiol. Methods* 69:381–383.
13. Franzolin, M. R., R. C. Alves, R. Keller, T. A. Gomes, L. Beutin, and M. L. Barreto. 2005. Prevalence of diarrheagenic *Escherichia coli* in children with diarrhea in Salvador, Bahia, Brazil. *Mem. Inst. Oswaldo Cruz* 100:359–363.
14. Gannon, V. P. J., S. D'Souza, T. Graham, R. K. King, K. Rahn, and S. Read. 1997. Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *J. Clin. Microbiol.* 35:656–662.
15. Hammar, M., Z. Bian, and S. Normark. 1996. Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 93:6562–6566.
16. He, Y., J. E. Keen, R. B. Westerman, E. T. Littledike, and J. Kwang. 1996. Monoclonal antibodies for detection of the H7 antigen of *Escherichia coli*. *Appl. Environ. Microbiol.* 62:3325–3332.
17. Hidalgo, G., X. Chen, A. G. Hay, and L. W. Lion. 2010. Curli produced by *Escherichia coli* PHL628 provide protection from Hg (II). *Appl. Environ. Microbiol.* 76:6939–6941.
18. Hou, S., Z. Liu, A. W. Young, S. L. Mark, N. R. Kallenbach, and D. Ren. 2010. Effects of Trp- and Arg-containing antimicrobial-peptide structure on inhibition of *Escherichia coli* planktonic growth and biofilm formation. *Appl. Environ. Microbiol.* 76:1967–1974.
19. Houari, A., and P. Di Martino. 2007. Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation. *Lett. Appl. Microbiol.* 45:652–656.
20. Hrudey, S. E., P. Payment, P. M. Huck, R. W. Gillham, and E. J. Hrudey. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Sci. Technol.* 47:7–14.
21. Hu, Y., Q. Zhang, and J. C. Meitzler. 1999. Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by multiplex PCR. *J. Appl. Microbiol.* 87:867–876.
22. Kassenborg, H. D., C. W. Hedberg, M. Hoekstra, M. C. Evans, A. E. Chin, R. Marcus, D. J. Vugia, K. Smith, S. D. Ahuja, L. Slutsker, P. M. Griffin, and the Emerging Infections Program FoodNet Working Group. 2004. Farm visits and undercooked hamburgers as major risk factors for sporadic *Escherichia coli* O157:H7 infection: data from a case-control study in 5 FoodNet sites. *Clin. Infect. Dis.* 38(Suppl. 3): S271–S278.
23. Keen, J. E., T. E. Wittum, J. R. Dunn, J. L. Bono, and L. M. Durso. 2006. Shiga-toxigenic *Escherichia coli* O157 in agricultural fair livestock, United States. *Emerg. Infect. Dis.* 12:780–786.
24. Lomander, A., P. Schreuders, E. Russek-Cohen, and L. Ali. 2004. Evaluation of chlorine's impact on biofilms on scratched stainless steel surfaces. *Bioresour. Technol.* 94:275–283.
25. Marouani-Gadri, N., G. Augier, and B. Carpentier. 2009. Characterization of bacterial strains isolated from a beef-processing plant following cleaning and disinfection—influence of isolated strains on biofilm formation by Sakaï and EDL 933 *E. coli* O157:H7. *Int. J. Food Microbiol.* 133:62–67.
26. Marouani-Gadri, N., D. Chassaing, and B. Carpentier. 2009. Comparative evaluation of biofilm formation and tolerance to a chemical shock of pathogenic and nonpathogenic *Escherichia coli* O157:H7 strains. *J. Food Prot.* 72:157–164.
27. Marouani-Gadri, N., O. Firmesse, D. Chassaing, D. Sandris-Nielsen, N. Arneborg, and B. Carpentier. 2010. Potential of *Escherichia coli* O157:H7 to persist and form viable but non-culturable cells on a food-contact surface subjected to cycles of soiling and chemical treatment. *Int. J. Food Microbiol.* 144:96–103.
28. Misyurina, O., D. J. Asper, W. Deng, B. B. Finlay, D. Rogan, and A. A. Potter. 2010. The role of Tir, EspA, and NleB in the colonization of cattle by Shiga toxin producing *Escherichia coli* O26:H11. *Can. J. Microbiol.* 56:739–747.
29. Mora, A., M. Blanco, J. E. Blanco, G. Dahbi, C. López, and P. Justel. 2007. Serotypes, virulence genes and intimin types of Shiga toxin (verocytotoxin)-producing *Escherichia coli* isolates from minced beef in Lugo (Spain) from 1995 through 2003. *BMC Microbiol.* 7:13.
30. Mosteller, T. M., and J. R. Bishop. 1993. Sanitizer efficacy against attached bacteria in a milk biofilm. *J. Food Prot.* 56:34–41.
31. Paton, A. W., and J. C. Paton. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA,* enterohemorrhagic *E. coli hlyA,* rfb_{O111}, and rfb_{O157}. *J. Clin. Microbiol.* 36:598–602.
32. Possé, B., L. De Zutter, M. Heyndrickx, and L. Herman. 2008. Novel differential and confirmation plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. *FEMS Microbiol. Lett.* 282:124–131.
33. Possé, B., L. De Zutter, M. Heyndrickx, and L. Herman. 2008. Quantitative isolation efficiency of O26, O103, O111, O145 and O157 STEC serotypes from artificially contaminated food and cattle faeces samples using a new isolation protocol. *J. Appl. Microbiol.* 105:227–235.
34. Prére, M. F., S. C. Bacrie, O. Baron, and O. Fayet. 2006. Bacterial aetiology of diarrhoea in young children: high prevalence of enteropathogenic *Escherichia coli* (EPEC) not belonging to the classical EPEC serogroups. *Pathol. Biol. (Paris)* 54:600–602.
35. Prigent-Combaret, C., G. Prensier, T. T. L. Thi, O. Vidal, P. Lejeune, and C. Dorel. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains: role of flagella, curli and colanic acid. *Environ. Microbiol.* 2:450–464.
36. Rivera-Betancourt, M., and J. E. Keen. 2000. Murine monoclonal antibodies specific for lipopolysaccharide of *Escherichia coli* O26 and O111. *Appl. Environ. Microbiol* 66:4124–4127.
37. Ryu, J. H., and L. R. Beuchat. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effect of exopolysaccharide and Curli production on its resistance to chlorine. *Appl. Environ. Microbiol.* 71:247–254.
38. Saldaña, Z., J. Xicohtencatl-Cortes, F. Avelino, A. D. Phillips, J. B. Kaper, J. L. Puente, and J. A. Girón. 2009. Synergistic role of curli and cellulose in cell adherence and biofilm formation of attaching and effacing *Escherichia coli* and identification of Fis as a negative regulator of curli. *Environ. Microbiol.* 11:992–1006.
39. Silagyi, K., S. H. Kim, Y. M. Lo, and C. I. Wei. 2009. Production of biofilm and quorum sensing by *Escherichia coli* O157:H7 and its transfer from contact surfaces to meat, poultry, ready-to-eat deli, and produce products. *Food Microbiol.* 26:514–519.

40. Stopforth, J. D., J. Samelis, J. N. Sofos, P. A. Kendall, and G. C. Smith. 2003. Influence of organic acid concentration on survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in beef carcass wash water and on model equipment surfaces. *Food Microbiol.* 20:651–660.
41. Torres, A. G., R. C. Vazquez-Juarez, C. B. Tutt, and J. G. Garcia-Gallegos. 2005. Pathoadaptive mutation that mediates adherence of shiga toxin-producing *Escherichia coli* O111. *Infect. Immun.* 73: 4766–4776.
42. Tristão, L. C., A. G. Gonzalez, C. A. Coutinho, A. M. Cerqueira, M. J. Gomes, K. Irino, B. E. Guth, and J. R. Andrade. 2007. Virulence markers and genetic relationships of Shiga toxin-producing *Escherichia coli* strains from serogroup O111 isolated from cattle. *Vet. Microbiol.* 119:358–365.
43. Uhlich, G. A., J. E. Keen, and R. O. Elder. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 67:2367–2370.
44. Uhlich, G. A., D. P. Rogers, and D. A. Mosier. 2010. *Escherichia coli* serotype O157:H7 retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. *Foodborne Pathog. Dis.* 7:935–943.
45. U.S. Department of Agriculture, Food Safety and Inspection Service. 2011. Shiga toxin-producing *Escherichia coli* in certain raw beef products. *Fed. Regist.* 76:58157–58165.
46. Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. *J. Bacteriol.* 180:2442–2449.
47. Werber, D., A. Fruth, A. Liesegang, M. Littmann, U. Buchholz, and R. Prager. 2002. A multistate outbreak of Shiga toxin-producing *Escherichia coli* O26:H11 infections in Germany, detected by molecular subtyping surveillance. *J. Infect. Dis.* 186:419–422.
48. Westerman, R. B., Y. He, J. E. Keen, E. T. Littledike, and J. Kwang. 1997. Production and characterization of monoclonal antibodies specific for the lipopolysaccharide of *Escherichia coli* O157. *J. Clin. Microbiol.* 35:679–684.
49. Whittam, T. S. 1998. Evolution of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains, p. 195–209. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. ASM Press, Washington, DC.
50. Yu, J., D. Kim, and T. Lee. 2010. Microbial diversity in biofilms on water distribution pipes of different materials. *Water Sci. Technol.* 61: 163–171.