**Curli fimbriae are conditionally required in *Escherichia coli* O157:H7 for
initial attachment and biofilm formation**

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ABSTRACT

Several species of enteric pathogens produce curli fimbriae, which may affect their interaction with surfaces and other microbes in nonhost environments. Here we used two *E. coli* O157:H7 outbreak strains with distinct genotypes to understand the role of curli in surface attachment and biofilm formation in several systems relevant to fresh produce production and processing. Curli significantly enhanced the initial attachment of *E. coli* O157:H7 to spinach leaf and stainless steel surfaces by 5-fold. Curli was also required for *E. coli* O157:H7 biofilm formation on stainless steel and enhanced biofilms on glass by 19-27 fold in LB no-salt broth. However, this contribution was not observed when cells were grown in sterile spinach lysates. Furthermore, both strains of *E. coli* O157:H7 produced minimal biofilms on polypropylene in LB no-salt broth but considerable amounts in spinach lysates. Under the latter conditions, curli appeared to slightly increase the biofilm production. Notably, curli played an important role in the formation of mixed biofilm by *E. coli* O157:H7 and plant-associated microorganisms in spinach leaf washes, as revealed by confocal microscopy. Little or no *E. coli* O157:H7 biofilms were detected at 4°C, supporting the importance of temperature control in postharvest and produce processing environments.

Keywords:

Escherichia coli O157:H7, foodborne pathogen, curli fimbriae, attachment, biofilm, spinach, plant, produce, stainless steel, glass, surface

1. INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), primarily the serotype O157:H7, is one of the main bacterial causal agents of foodborne illness outbreaks associated with fresh produce (Rangel et al., 2005). STECs can survive in nonhost environments for prolonged periods of time, thus posing a potential risk for public health. Numerous studies have examined the potential sources of produce contamination in both pre- and postharvest environments. In the field, contaminated water, dust containing manures, wildlife animals, and insects can potentially serve as the sources of produce contamination (Berger et al., 2010; Brandl, 2006; Cooley et al., 2014; Strawn et al., 2013). At the postharvest level, produce contamination may result from water used for washing and processing produce, workers, or cross-contamination from other food (Beuchat and Ryu, 1997; Gomez-Lopez et al., 2013; Lynch et al., 2009). Additionally, inadequate storage temperature (Alegre et al., 2010; Sim et al., 2013) or minimally processed vegetables such as precut vegetables may facilitate amplification of pathogens on produce (Brandl, 2008). Because produce such as salads and fruits are often consumed raw, contamination of produce by enteric pathogens poses a high health risk to consumers.

Biofilm production is an ubiquitous lifestyle of bacteria associated with a surface and is generally believed to be advantageous as it provides bacteria metabolic benefits and physical protection in harsh environments and accelerates genetic exchanges (Watnick and Kolter, 2000). Biofilm-associated cells are more resistant to many toxic substances including antibiotics, chlorine, and detergents, thus they are generally more resistant to antimicrobial washes used in the produce industry (Costerton et al., 1987; De Beer et al., 1994; Ryu and Beuchat, 2005; Suci et al., 1994; Uhlich et al., 2006). Biofilms of enteric pathogens have been observed for STEC, *Salmonella*, *Campylobacter*, *Listeria* and *Shigella* on a variety of fruits and vegetables including

tomatoes, melons and parsley, cilantro, and lettuce and spinach as well as on various abiotic surfaces (Annous et al., 2005; Brandl and Mandrell, 2002; Iturriaga et al., 2007; Warner et al., 2008), which may serve as a source of cross-contamination in postharvest and food-processing environments.

Surface attachment is the first step for bacteria to establish biofilm. Enteric pathogens produce an array of adhesive structures and proteins for colonization of their animal hosts (McWilliams and Torres, 2014). Many of these adhesion factors are also important for STEC to colonize plants or produce biofilms on abiotic surfaces. Such adhesion factors include curli fimbriae, flagella, cellulose, LPS, colanic acid, and several outer membrane proteins. Curli are the long aggregative fimbriae that are commonly present in many *Enterobacteriaceae* (Zogaj et al., 2003). It was first discovered in *E. coli* and described as an adhesin due to its specific interaction with human host proteins (Hammar et al., 1995; Olsen et al., 1989). Unlike pili or flagella, curli are functional amyloids. The major curli subunit protein CsgA is capable of self-polymerization into a β -sheet-rich amyloid fiber, and as a result, curli fibers are highly aggregative and stable (Chapman et al., 2002). Recent studies suggested that curli mediate adherence of *E. coli* cells to and internalization of bacterial cells by eukaryotic cells, although this function appeared to be cell-line dependent (Gophna et al., 2001; Gophna et al., 2002; Uhlich et al., 2002). In the absence of long polar fimbriae, curli appeared to play a role in the intestinal colonization of *E. coli* O157:H7 in rabbit (Lloyd et al., 2012). Interestingly, the expression of curli genes is responsive to many environmental factors such as temperature below 30°C (Barnhart and Chapman, 2006), implying an ecological role of this unique adhesin in nonhost environments. Indeed, curli are implicated in the attachment to, or biofilm formation of, enteric pathogens on abiotic surfaces (Goulter et al., 2010; Goulter-Thorsen et al., 2011; Ryu et

al., 2004) or plant tissues (Barak et al., 2005; Torres et al., 2005); however, this contribution appears to be strain-dependent, which in part is due to a complex regulatory network governing curli biogenesis. Natural variation in curli production has been reported for *E. coli* isolates originating from diverse environments (Maurer et al., 1998; Szabo et al., 2005) as well as in STEC O157:H7 populations (Carter et al., 2011). Increased curli biogenesis has been linked to mutations in the promoter of *csgD*, encoding a positive regulator of curli genes *csgA* and *csgB* (Uhlich et al., 2001), in *rscB*, encoding the response regulator of two-component signal transduction system RcsCDB (Carter, Parker, et al., 2012), or in *rpoS*, encoding the alternate sigma factor 38 (Carter et al., 2014).

We previously characterized natural curli variants in three distinct genotypes of *E. coli* O157:H7 (Carter et al., 2011). Group I includes variants isolated from the 2006 spinach associated-outbreak strains; group II includes variants isolated from a 2006 bagged iceberg lettuce-associated outbreak strains, and group III includes variants isolated from the 1993 hamburger-associated outbreak strains. Curli-expressing variants in group II and III produce considerable amounts of curli fimbriae at 26°C based on the pigmentation of colonies grown on Congo Red agar plates and on colony hybridization with antibody against the curli major subunit protein CsgA (Carter et al., 2011). In this study, we used both group II and III curli-expressing variants and their isogenic curli-deletion mutants to systematically investigate the role of curli fimbriae in initial attachment and biofilm formation by *E. coli* O157:H7 in several systems relevant to produce postharvest and processing environments.

2. MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The *E. coli* strains and mutants used in this

study and their sources are listed in Table 1. Strains were grown routinely in LB broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) at 37°C, or LB-no salt (LBNS) broth at 26°C, a condition known to induce curli biogenesis. Antibiotics were used at the following concentrations: kanamycin (Km), 25 µg ml⁻¹; gentamicin (Gm), 15 µg ml⁻¹; and rifampicin (Rif), 25 µg ml⁻¹ for maintenance and 100 µg ml⁻¹ for selection.

Preparation of spinach lysates. Fresh spinach leaf lysates were prepared on the day of the experiment from bagged ready-to-eat organic baby spinach as described previously (Kyle et al., 2010). Briefly, the spinach leaves were homogenized using an Omega 8003 juicer (Omega Products, Inc., Harrisburg, PA). The homogenized sample was centrifuged twice at 20,000 g for 10 min to pellet the plant debris. The supernatant, hereafter called spinach lysate, was filter-sterilized and used as a medium for biofilm formation. Sterile lysates were prepared by first filtering through a 0.45 µm-pore-size filter, and then through a 0.2 µm-pore-size filter. The sterility of the lysates was verified by plating 100 µl of filtered lysates onto a LB agar plate and incubating the plate at 26°C for at least two days to test for the presence of colonies.

Biofilm formation and quantification. Biofilm experiments were carried out as described previously (Carter et al., 2011) with minor modifications. Briefly, single colonies of each *E. coli* O157:H7 strains and their isogenic curli-deletion mutants were inoculated in LBNS broth supplemented with the appropriate antibiotic and grown at 26°C overnight on a shaker (150 rpm). Cells were collected by centrifugation at 8,000 g for 3 min, washed twice in potassium phosphate buffer (10 mM, pH 7.0) (KP buffer), and resuspended in KP buffer for inoculation. Biofilm formation on glass and plastic surfaces was carried out by aliquoting one ml of LBNS broth or spinach lysates inoculated with either 5x10³ or 5x10⁵ cells/ml into a borosilicate glass tube or a polypropylene culture tube and incubating statically at 26°C for 2 or 5 days. After

incubation, the planktonic cells were removed carefully, and the tubes were rinsed twice with one ml sterile distilled water and then stained with one ml 0.1% crystal violet at room temperature for 30 min. The dye was then removed gently, and the tubes were washed twice with sterile distilled water. The crystal violet bound to the glass tube was solubilized in one ml of 33% acetic acid and the absorbance was determined at 570 nm using a microplate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA). Uninoculated media served as controls. Each data set was the average of results from at least five biological replicates. For cells inoculated into spinach lysates, biofilm formation on abiotic surfaces at 4°C was examined also.

Biofilm formation on stainless steel surfaces was carried out as previously described (Carter, Xue, et al., 2012). Precut stainless steel coupons (1.0 x 0.5 inch) (SSC) were purchased from Speedy metals LLC and cleaned as described previously (Rivas et al., 2007). Briefly, coupons were soaked in acetone (Sigma) for 30 minutes to degrease, followed by rinsing with distilled water to remove any traces of acetone. The coupons were then soaked in alkaline detergent for one hour at 70°C, followed by thorough rinsing with distilled water to remove any trace of detergent. The coupons were rinsed once more with Millipore water and air-dried prior to autoclaving. For biofilm formation, a sterile coupon was placed in a culture tube containing two ml of LBNS broth or spinach lysates inoculated with 5×10^3 or 5×10^5 cells/ml, and incubated statically at 26°C for 5 days, or at 4°C for 2 weeks. At various incubation times, coupons were removed and rinsed twice with sterile water, and placed on a 24-well plate until dried. The coupons were then transferred to a culture tube containing two ml of 0.1% CV and stained at room temperature for 30 min, followed by rinsing twice with water. Quantification of CV on the coupon was carried out as described above. Coupons placed in uninoculated LBNS or spinach lysates served as controls.

Attachment to spinach leaves. Rifampicin resistant mutants of both wild-type *E. coli* O157:H7 and their corresponding curli-deletion mutants were used in spinach leaf attachment assays. Supplementation of 100 µg/ml of rifampicin in LB agar plates was shown to be effective in inhibiting the growth of natural microflora on the baby spinach under the conditions used in this study. Single colonies of each strain and mutant were grown on LBNS agar plates supplemented with 25µg/mL rifampicin at 26°C for 2-3 days to induce the curli biogenesis. The cells were removed from the plates using a cotton swab and resuspended in five mL KP buffer. The cells were then used for inoculation. Whole spinach leaves purchased from a local retail store were submerged in a plant culture box (Magenta™ GA-7 Vessel, Sigma-Aldrich) containing the cell suspension at a final concentration of 10⁶ cells/ml and incubated at 26°C for 3 h with gentle rotation. The actual concentration of cell suspension was determined by enumerating the colony forming units (CFUs) on LB agar plates supplemented with 100 µg/ml rifampicin. At the end of the incubation, each leaf was washed twice in sterile KP buffer by completely submerging the leaf in washing buffer, each for 30 seconds to release loosely attached cells. Two discs (one on each side of the main leaf vein) were cut with a #6 borer and homogenized in 250 µl of KP buffer. The total number of viable pathogen cells on the spinach discs was determined by enumerating colonies on LB agar plates supplemented with 100 µg/ml rifampicin. Spinach leaves immersed in uninoculated KP buffer served as controls.

Competitive attachment assay. Organic baby spinach was purchased from a local retail store on the day of the experiment to make spinach leaf wash water. Approximately 50 g of organic baby spinach leaves were placed in a Ziplock bag containing 250 ml KP buffer. The leaves were sonicated in an ice-water bath for 10 min followed by incubation on a Belly Dancer® undulating orbital shaker at room temperature for 15 min. The leaf wash water was

then filtered through a double layer of cheesecloth to remove plant debris. The filtrate, hereafter called spinach leaf wash water, was used for pathogen inoculation and biofilm formation. Cell suspensions were prepared as described above for spinach leaf attachment and used to inoculate leaf wash water. A sterile coupon was placed in a culture tube containing two ml of leaf wash water inoculated with 10^6 cells/ml of *E. coli* O157:H7 wild-type or curli-deletion mutant. The actual cell concentrations were determined by enumerating colonies on LB agar plates containing 100 µg/ml of Rifampicin. The coupons were incubated at 26°C statically for 3 h. The coupon was then removed, rinsed twice with sterile KP buffer and placed in a new culture tube containing one ml KP buffer. Cells attached to the coupon were released into the buffer by sonication and vortex as described above. The population size of *E. coli* O157:H7 on SSC was determined by enumerating colonies on LB agar plates containing 100 µg/ml of Rifampicin.

Confocal microscopy. The spinach leaf wash water and the bacterial inoculum were prepared as described above for the competitive attachment assay. A sterile borosilicate glass cover slip or SSC was placed in a culture tube containing five ml of leaf wash water inoculated with 10^6 cells/ml of the *E. coli* O157:H7 wild-type or curli-deletion mutant strains transformed with pGT-Kan (Brandl et al., 2005), which allows for the constitutive expression of the green fluorescent protein (GFP) gene. The tubes were incubated at 26°C under stationary conditions for six days. At the end of incubation, the glass cover slip or SSC was removed from the culture tube and washed twice prior to being mounted on a glass slide. To visualize the leaf-associated microorganisms, the cover slips or SSC were stained with the cell-permeant SYTO® 62 red fluorescent nucleic acid dye (Life Technologies) at a final concentration of 25 µM at room temperature for 10 min, followed by two 10-min washes by immersion in water. The mixed biofilms were visualized with a Leica confocal microscope TCS SP5 (Leica Microsystems,

Germany). Pseudo-three-dimensional (pseudo-3D) images were constructed by projected series of multiple optical scans in the z plane as described previously (Brandl and Huynh, 2014). Fluorescence-based imaging of bacteria on the SSC was not possible due to the large amount of laser light reflected off the stainless steel surface.

Statistical analysis. Statistical analysis was computed with SigmaPlot version 11.0 (Systat Software, Inc.). An unpaired t-test was performed for a two-group comparison and the Analysis of Variance (ANOVA) followed by Bonferroni t-test was performed for multiple comparisons. For comparison of data sets for which the equal variance test failed, a Mann-Whitney Rank Sum test was performed.

3. RESULTS

Contribution of curli to *E. coli* O157:H7 biofilm formation on abiotic surfaces. We first evaluated the biofilm formation of *E. coli* O157:H7 strains and their corresponding curli-deletion mutants on different abiotic surfaces when cells were grown in LBNS broth at 26°C, a condition known to induce the biogenesis of curli fimbriae. Given that the biofilm population of an *E. coli* O157:H7 strain would be determined partly by its surface attachment proficiency and the growth rate under the biofilm-forming conditions, we first compared the growth of *E. coli* O157:H7 wild-type strains with their corresponding curli-deletion mutant in LBNS broth at 26°C. No significant difference in growth was observed for any pairs of wild-type strain and the curli-deletion mutant (Data not shown).

Among the three abiotic surfaces examined (stainless steel, glass, and polypropylene), the greatest biofilms were observed on the stainless steel coupon (SSC) for both *E. coli* O157:H7 strains MQC17 and MQC21 (Fig. 1). Biofilm formation on SSC was completely abolished in

both curli-deletion mutants under the conditions examined since regardless of initial inoculation concentration, there was no significant difference in biofilm production between the control (no pathogen) and any of the two curli-deletion mutants (Fig. 1, A and B). Furthermore, strain MQC21 produced significantly greater amounts of biofilm on SSC than the strain MQC17 under the conditions examined (low inoculum, 48 h, t test $P=0.003$; low inoculum, 120 h, t test $P<0.001$; high inoculum, 48 h, t test $P<0.001$; high inoculum, 120 h, t test $P<0.001$).

Both *E. coli* O157:H7 strains produced considerable amount of biofilm on glass surfaces at 48 h and 120 h following inoculation at 10^3 cells/ml (Fig. 1, C) or 10^5 cells/ml (Fig. 1, D). Although there was no difference at 48 h between the wild-type strains and their isogenic curli-deletion mutant, a significant reduction was observed in both curli-deletion mutants compared with their corresponding wild-type strain at 120 h following inoculation at both concentrations. Similarly to SSC, strain MQC21 generally produced greater biofilms on glass surfaces than strain MQC17. A significant higher amount of MQC21 biofilm was observed at 48 h with the high inoculation concentration (Fig. 1, D), and at 120 h for both inoculation concentrations (Fig. 1, C and D) (t test, $P<0.05$). Thus, similarly to SSC, curli contributed to biofilm formation by *E. coli* O157:H7 on a glass surface. However, unlike SSC, curli did not appear to be required in *E. coli* O157:H7 for the biofilm formation on glass surfaces since curli-deletion mutants of the both strains produced considerable amounts of biofilm on glass surfaces under the conditions examined in this study (significantly higher than controls).

Unlike on SSC and glass surfaces, both *E. coli* O157:H7 strains produced little or no biofilms on polypropylene surfaces (Fig. 1, E and F). At 48 h after inoculation, there was no significant difference in biofilm formation between the wild-type strains and their corresponding curli-deletion mutants under any of the conditions. Biofilms on polypropylene surfaces were

detected for both *E. coli* O157:H7 wild-type strains at 120 h following inoculation (significantly higher than the control); however, the amount of biofilm was not significantly different between the curli-deletion mutants and their parental strains.

Role of curli in biofilm formation on abiotic surfaces in spinach lysates. Because bacterial biofilm formation is largely dependent on the physiological state of the cells, we examined the biofilms of *E. coli* O157:H7 and the curli-deletion mutants on abiotic surfaces when cells were grown in sterile spinach lysates. At 26°C, no significant difference was observed in the growth rate between any pair of the wild-type strain and its isogenic curli-deletion mutant when cells were inoculated in sterile spinach lysates; at 4°C, no growth was observed for any of wild-type and mutant strains since their population sizes remained constant during the 14 d incubation (data not shown). Biofilms on SSC were detected for both *E. coli* O157:H7 strains at 26°C following 24 h incubation (Fig. 2, A); however, there was no difference between the wild-type strains and their corresponding isogenic curli-deletion mutants at any of the incubation time examined (24 h, 48 h, and 120 h). For both strains, greater biofilms were detected with longer incubation times. In contrast to 26°C, the biofilm on SSC at 4°C was indistinguishable from the control group (no pathogen inoculation) for any of *E. coli* O157:H7 strains and their curli-deletion mutants at either of the incubation time (5 d, 7 d, and 14 d) examined in this study (Fig. 2, B).

Similarly to SSC, when cells were inoculated in spinach lysates and incubated at 26°C, biofilms for both strains and both curli-deletion mutants were detected on glass surfaces as early as 24 h after incubation (Fig. 2, C). There was no difference between the wild-type strains and their curli-deletion mutants at any of the incubation times examined. Furthermore, similarly to

SSC, both *E. coli* O157:H7 and their curli-deletion mutants produced little biofilm on glass surfaces when cells were incubated at 4°C (Fig. 2, D).

Unlike cells grown in LBNS broth, biofilms on polypropylene surfaces were detectable (significantly greater than the control) as early as 24 h following inoculation when cells were grown in spinach lysates at 26°C (Fig. 2, E). At 120 h, biofilms for both wild-type strains were the greatest among the three time points examined and comparable to those on SSC (Fig. 2, A) and glass surfaces (Fig. 2, C). Furthermore, at 120 h, curli appeared to contribute but were not required for biofilm formation on polypropylene since a significant reduction was observed for both curli-deletion mutants compared with their parental strains (t test, $P < 0.05$). Similarly to SSC and glass surfaces, little biofilm was produced for any of the strains and mutants when cells were incubated at 4°C (Fig. 2, F).

Role of curli fimbriae in surface attachment. Surface attachment is the first step for bacteria to establish a biofilm. Therefore, we further evaluated the role of curli fimbriae in initial attachment of *E. coli* O157:H7 cells to whole baby spinach leaves. Following 3 h incubation at 26°C, the population sizes of the wild-type strains and their curli-deletion mutants on each spinach leaf were compared. In the lettuce isolate (MQC57), the population size of the curli-deletion mutant (MQC946) on the spinach leaves was reduced more than five-fold compared with the wild-type strain (MQC57) (Fig. 3). Similarly, in the clinical *E. coli* O157:H7 isolate (MQC65), the population size of the curli-deletion mutant (MQC948) was reduced about four-fold compared with the wild-type strain MQC65 (Fig. 3).

We then evaluated the contribution of curli fimbriae to the attachment of *E. coli* O157:H7 to stainless steel, a common material used in the food industry, when *E. coli* O157:H7 was in the presence of the natural spinach leaf microflora. After 3 h of incubation at 26°C, the population of

the curli-deletion mutant on SSC was 20.6% and 25.5% that of the corresponding wild-type strain for lettuce isolate (MQC57) and human isolate (MQC65), respectively (Fig. 4). Thus, similarly to our results in the spinach leaf attachment assay, curli fimbriae in *E. coli* O157:H7 enhanced the attachment of pathogen cells to SSC. The population of the curli-deletion mutant cells attached to the stainless steel coupons was 4.2×10^4 and 5.1×10^4 cfu/coupon for MQC946 and MQC948, respectively, suggesting that curli were not strictly required for the SSC-attachment under the condition examined in this study.

Role of curli in formation of mixed biofilms. We further investigated the role of curli in the formation of mixed biofilms of *E. coli* O157:H7 and spinach leaf-associated indigenous microorganisms by confocal microscopy. Many large aggregates of the *E. coli* O157:H7 wild type strain were observed on the surfaces of glass cover slips and SSCs and some of the aggregates associated with debris from plant tissue and red autofluorescent bacteria (Fig. 5, A). Staining with the cell-permeant DNA dye SYTO® 62 confirmed that *E. coli* O157:H7 on the glass surfaces was primarily associated with spinach leaf indigenous bacteria, forming various sizes of mixed aggregates (Fig. 5, B). In contrast, many fewer curli-deletion mutant cells (Fig. 5, C) and aggregates were observed on the glass cover slips compared with the wild-type strain. Staining with SYTO® 62 revealed that the curli-deletion mutant mainly existed as individual cells when present in mixed aggregates (Fig. 5, D).

4. DISCUSSION

Biofilm formation on abiotic surfaces may enhance environmental survival of enteric pathogens and serve as a source of microbial contamination. Under *in vitro* condition (growth in culture media), curli appears to mediate cell-to-surface and cell-to-cell interactions during

biofilm formation on polystyrene (Prigent-Combaret et al., 2000) and serves as a main pellicle biofilm constituent in uropathogenic *E. coli* (UPEC) (Hung et al., 2013) and in *E. coli* O157:H7 (Uhlich et al., 2006). Our studies demonstrate that biofilm formation by *E. coli* O157:H7 is dependent on surface properties as well as environmental conditions. Strong biofilm formation was observed for both *E. coli* O157:H7 wild-type strains on SSC and borosilicate glass, but little or no biofilm was observed on polypropylene. This difference may have been caused by the hydrophobic nature of the polypropylene surfaces. Our results also indicate that contribution of curli to biofilm formation on abiotic surfaces is conditional and dependent on the physical properties of the surface. Under the *in vitro* conditions in this study, curli was required for biofilm formation on SSC but not on the glass surfaces, although curli enhanced biofilm production on the glass surfaces significantly. Contribution of curli to biofilm formation on both SSC and glass surfaces was completely abolished when cells were grown in spinach lysates, suggesting that the physiological state of *E. coli* O157:H7 is altered in plant-derived material in a way that affects its ability to develop biofilms via curli production. It is possible also that other adhesins play a more central role in the interaction of *E. coli* O157:H7 with these surfaces since Kyle et al. reported the upregulation of several fimbriae in this pathogen upon exposure to lettuce lysates (Kyle et al., 2010). Additionally, in spinach lysate, both *E. coli* O157:H7 strains produced a considerable amount of biofilm on the polypropylene surfaces, which was comparable to the biofilms measured on SSC and glass surfaces. It is unclear whether this difference is due solely to a change in *E. coli* O157:H7 physiology or also a result of chemical components in the spinach lysates that may affect the interaction of the bacterial cells with that abiotic surface.

Biofilm formation by *E. coli* O157:H7 on abiotic surfaces was additionally affected greatly by temperature. When cells were inoculated in LBNS broth and incubated at 4°C, no

biofilms for either of the two wild-type *E. coli* O157:H7 strains were detected on any of the abiotic surfaces examined in this study (Data not shown). Similarly, when cells were grown in spinach lysates and incubated at 4°C, little or minimal biofilms were detected for both *E. coli* O157:H7 strains.

Attachment is the first step for bacteria to establish colonization. Bacterial attachment results from complex interactions among planktonic bacteria, surface-associated bacteria, and the surface, and is affected by local physico-chemical conditions and surface structure. Therefore, the role of curli in surface attachment and subsequently colonization could vary largely. Indeed, it has been reported that the contribution of curli to initial attachment in *E. coli* O157:H7 is dependent on strain, growth medium, and abiotic surface. Curli appeared to facilitate the attachment of *E. coli* O157:H7 to glass surfaces in most growth media tested, including Luria Bertani broth (LB) and nutrient broth (NB), enhance the attachment to steel surfaces in LB but modulate the attachment in NB (Goulter-Thorsen et al., 2011). The role of curli in attachment of enteric pathogen to produce surfaces has been investigated in several studies. But due to large differences in attachment assay and strains used in the studies, contradictory findings have been reported. Results obtained in studies using curli variants rather than isogenic curli mutants must be interpreted with caution since it is known that mutations in several genes encoding global transcriptional regulators such as *rpoS* and *rcsB* are common in natural curli variants (Carter et al., 2014; Carter, Parker, et al., 2012).

Attachment of *S. enterica* Enteritidis to alfalfa sprouts appeared to be dependent on the minor curli subunit protein CsgB but not the curli subunit protein CsgA, suggesting that at least, the nucleator protein CsgB facilitated the interaction of *Salmonella* with sprout tissue (Barak et al., 2005). In *E. coli* O157:H7, curli appeared to enhance the attachment of *E. coli* O157:H7 to

lettuce leaves when cells were spot inoculated on the leaves, followed by examining the tightly attached cells through different leaf-washing steps (Fink et al., 2012). However, there was no difference in cell population attached to sprouts between wild-type and *csgA*-deletion mutant, implying that curli is not required in *E. coli* O157:H7 for attachment and/or colonization on sprouts (Torres et al., 2005). Our results suggested that curli fimbriae in *E. coli* O157:H7 facilitated the initial attachment of pathogen cells to whole spinach leaf, which support a role for curli in attachment of *E. coli* O157:H7 to plant tissue as reported previously in lettuce (Fink et al., 2012) and spinach (Macarisin et al., 2012; Saldana et al., 2011). Since a considerable amount of curli-deletion mutant cells were detected on the spinach leaf (about 10^4 cells per spinach disc), other factors in *E. coli* O157:H7 evidently mediated the attachment. Similarly, curli fimbriae also promote competitive attachment of *E. coli* O157:H7 to abiotic surface in the presence of plant-associated microorganisms. Unlike in sterile buffer, the attachment of *E. coli* O157:H7 to SSC in spinach washing water would be a result of interactions among pathogen cells, abiotic surface, and spinach leaf-associated microorganisms including bacteria that are known to be proficient in biofilm formation such as *Pseudomonas* spp. (Carter, Xue, et al., 2012).

Our results additionally reveal that curli fimbriae are involved in the development of mixed biofilms by *E. coli* O157:H7 and spinach-associated microorganisms on various abiotic surfaces. The participation of *E. coli* O157:H7 in mixed biofilms via these long aggregative fimbriae may mediate interactions of this human pathogen with the plant microflora and thereby contribute to stabilization of the mature biofilm as has been suggested in several studies (Brandl et al., 2011; Macarisin et al., 2013; Serra et al., 2013). In turn, the presence of plant-associated bacterial species in these consortia may increase the survival of *E. coli* O157:H7 under adverse conditions as was reported for *S. enterica* in mixed aggregates in the lettuce phyllosphere (Poza-

Carrion et al., 2013). Since pathogen cells within mixed-species biofilms are also more resistant to common antimicrobial compounds than cells within mono-species biofilms (Schwering et al., 2013; Wang et al., 2013), the formation of mixed biofilms by enteric pathogens in food or on food contact surfaces may pose a serious threat to public health. Our finding that curli fimbriae play an essential role in the formation of mixed biofilms by *E. coli* O157:H7 and spinach leaf-associated microflora suggests a putative target for disruption of such biofilms.

5. CONCLUSIONS

We used two *E. coli* O157:H7 outbreak strains with distinct genotypes and their curli-deletion mutants to understand the role of curli in surface attachment and biofilm maturation in several systems relevant to produce production and processing environments. Contribution of curli to biofilm formation on abiotic surfaces was conditional, depending on cell physiology, abiotic surface, and local physiochemical condition. In LBNS broth, *E. coli* O157:H7 produced strong biofilms on both SSC and glass, which were largely mediated by curli, but formed minimal biofilms on polypropylene. However, curli fimbriae were not required for *E. coli* O157:H7 biofilm formation on abiotic surfaces in sterile spinach lysates. Curli enhanced the initial attachment of *E. coli* O157:H7 to spinach leaves and stainless steel and contributed to the formation of mixed biofilms by *E. coli* O157:H7 and plant-associated bacterial species on abiotic surfaces in spinach-wash water. Such mixed biofilms may enhance the survival of *E. coli* O157:H7 to environmental stress and antimicrobials and serve as a contamination source in produce-processing environments.

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FIGURE LEGENDS

Figure 1. Biofilms of *E. coli* O157:H7 in growth medium. Biofilms of *E. coli* O157:H7 wild-type strains (MQC17 and MQC21) and their isogenic *csgA* deletion mutants (MQC114 and MQC118) on the stainless steel surfaces (A and B), glass surfaces (C and D), and plastic (polypropylene) surfaces (E and F) were determined as described in Methods and Materials section. At least five biological replicates were performed for each group. The difference in biofilm between wild-type and curli-deletion mutant, or between each sample and the control (without inoculation) were determined by the *P* value of t test (*, $P < 0.05$; **, $P < 0.001$).

Figure 2. Biofilms of *E. coli* O157:H7 in spinach lysates. Biofilms of *E. coli* O157:H7 wild-type strains (MQC17 and MQC21) and their isogenic *csgA* deletion mutants (MQC114 and MQC118) on the stainless steel surfaces (A and B), glass surfaces (C and D), and plastic (polypropylene) surfaces (E and F) were determined as described in Methods and Materials section. At least five biological replicates were performed for each group. The difference in biofilm between wild-type and curli-deletion mutant, or between each sample and the control (without inoculation) were determined by the *P* value of t test (*, $P < 0.05$; **, $P < 0.001$).

Figure 3. Attachment of *E. coli* O157:H7 to spinach leaf. The population sizes of *E. coli* O157:H7 wild-type strains (MQC57 and MQC65) and their isogenic *csgA* deletion mutants (MQC946 and MQC948) on the baby spinach leaves were determined as described in Methods and Materials. The attachment strength was expressed as the percent of total population on the

two leaf discs. At least five biological replicates were performed for each group. The difference between wild-type and mutant strains in leaf attachment was determined by the *P* value of t test.

Figure 4. Competitive attachment of *E. coli* O157:H7 and spinach-associated microflora to stainless steel surfaces. The population sizes of *E. coli* O157:H7 wild-type strains (MQC57 and MQC65) and their isogenic *csgA* deletion mutants (MQC946 and MQC948) attached to each SSC in the presence of spinach leaf-associated microflora were determined as described in Methods and Materials. The attachment strength was expressed as the percent of total population on the coupon. At least five biological replicates were performed for each group. The difference between wild-type and mutant strains in leaf attachment was determined by the *P* value of t test.

Figure 5. Biofilm formation by *E. coli* O157:H7 and spinach leaf-associated microflora on glass surfaces. Pseudo-3D confocal micrographs of GFP-labeled *E. coli* O157:H7 lettuce-associated outbreak strain MQC1038 (**A** and **B**) and its isogenic curli-deletion mutant MQC1039 (**C** and **D**) on glass cover slips incubated for six days in spinach leaf wash water. **A** and **C** are images of unstained GFP-labeled cells, whereas images in **B** and **D** show all cells that colonized the glass surfaces. Because of staining with the cell-permeant dye SYTO® 62 (**B** and **D**), the signal was assigned the pseudo-color red. Plant debris which was present in the leaf wash water and adhered to the glass, had autofluorescence mainly in the far red range and was assigned the pseudo-color blue in **A** and **C**. Scale Bars, 20 µm.

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602

603 **Table 1. Strains used in this study**

Strain/variant or plasmid	Antibiotic resistance ^a	Characteristics ^b	Source or Reference
<i>E. coli</i> O157:H7			
RM6535	None	A lettuce isolate linked to a bagged iceberg lettuce-associated outbreak in 2006	(Carter et al., 2011)
MQC17	None	Curli-expressing variant of RM6535 (RM6535R)	(Carter et al., 2011)
MQC114	Km ^R	<i>csgA</i> deletion mutant of RM6535R	This study
MQC57	Rif ^R	A spontaneous rifampicin resistant mutant of RM6535R	This study
MQC946	Rif ^R	A spontaneous rifampicin resistant mutant of MQC114	
RM6607	None	A human isolate linked to a hamburger-associated outbreak in 1993	(Carter et al., 2011)
MQC21	None	Curli-expressing variant of RM6607 (RM6607R)	(Carter et al., 2011)
MQC118	Km ^R	<i>csgA</i> deletion mutant of RM6607R	This study
MQC65	Rif ^R	A spontaneous rifampicin resistant mutant of RM6607R	This study
MQC948	Rif ^R	A spontaneous rifampicin resistant mutant of MQC118	This study
MQC1038	Gm ^R	MQC17 transformed with the <i>gfp</i> -expressing plasmid pGT-Kan	This study
MQC1039	Gm ^R	MQC114 transformed with the <i>gfp</i> -expressing plasmid pGT-Kan	This study

604 ^aKm^R, kanamycin resistance; Rif^R, rifampicin resistance; Gm^R, gentamicin resistance.