

# Role of Curli and Cellulose Expression in Adherence of *Escherichia coli* O157:H7 to Spinach Leaves

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## Abstract

Shiga-toxigenic *Escherichia coli* O157:H7 outbreaks have been linked to consumption of fresh produce. It is generally recognized that bacterial attachment to vegetal matrices constitutes the first step in contamination of fresh produce. Cellular appendages, such as curli fibers, and cellulose, a constituent of extracellular matrix, have been suggested to be involved in *E. coli* attachment and persistence in fresh produce. A comparative evaluation was conducted on the ability of Shiga toxin-producing *E. coli* O157:H7 strains EDL933 and 86-24, linked to two independent foodborne disease outbreaks in humans, and their mutants deficient in curli and/or cellulose expression to colonize and to firmly attach to spinach leaf. Inoculated spinach leaves were incubated at 22°C, and at 0, 24, and 48 h after incubation loosely and strongly attached *E. coli* O157:H7 populations were determined. Curli-expressing *E. coli* O157:H7 strains developed stronger association with leaf surface, whereas curli-deficient mutants attached to spinach at significantly ( $p < 0.01$ ) lower numbers. Attachment of cellulose-impaired mutants to spinach leaves was not significantly different from that of curliated strains. The relative attachment strength of *E. coli* O157:H7 to spinach increased with incubation time for the curli-expressing strains. Laser scanning confocal microscopy (LSCM) analysis of inoculated leaves revealed that curli-expressing *E. coli* O157:H7 were surrounded by extracellular structures strongly immunostained with anti-curli antibodies. Production of cellulose was not required to develop strong attachment to spinach leaf. These results indicate that curli fibers are essential for strong attachment of *E. coli* O157:H7 to spinach whereas cellulose is dispensable.

## Introduction

THE FOODBORNE OUTBREAKS OF *Escherichia coli* O157:H7 linked to consumption of fresh produce cause tremendous losses to the fresh produce industry and greatly damage consumer confidence in produce safety worldwide. In the United States, 54% of *E. coli* O157:H7 illnesses were attributed to contaminated produce (Scharff, 2010). It is generally recognized that bacterial attachment to plant surface constitutes the first step in contamination of fresh produce. Curli fimbriae and polysaccharide cellulose, two components of extracellular matrix of *E. coli*, promote bacterial attachment to cultured animal cells and intestinal epithelium, and may play a critical role in host colonization (Saldana *et al.*, 2009). Curli were also demonstrated to be involved in bacterial attachment to inert surfaces and together with cellulose contribute in cell to cell aggregation, biofilm development, and as a result increased resistance to chemical and physical removal (Cookson *et al.*, 2002; Ramey *et al.*, 2004; Ryu *et al.*, 2004a,b; 2005). Recent

studies have provided evidence that curli and cellulose also play a role in *E. coli* attachment to plant surfaces as well. For example, Torres *et al.* (2005) showed that curli-expressing strains of *E. coli* K12 manifest stronger attachment to alfalfa sprouts compared to those not producing curli; however, curli-deficient isogenic mutants of *E. coli* O157:H7 showed no differences in attachment to alfalfa sprouts compared to parental curli-producing genotype. Similarly, Jeter and Matthyse (2005) reported that K12 strains of *E. coli* (not producing curli) lacked the ability to adhere to plant surfaces, whereas the same strains carrying a plasmid encoding a gene for curli biosynthesis were able to attach to tomato and *Arabidopsis* seedlings, and alfalfa sprouts. In contrast, Boyer *et al.* (2007) suggested that curli are not essential for *E. coli* O157:H7 attachment to lettuce surfaces. Studies to address the role of cellulose in *E. coli* attachment to fresh produce also generated different results. For instance, Matthyse *et al.* (2008) demonstrated that *E. coli* O157:H7 mutants deficient in cellulose synthase had significantly lower binding ability to alfalfa

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sprouts comparing to the wild genotype, indicating the critical role of cellulose in binding and biofilm formation. In contrast, Uhlich *et al.* (2006) found that biofilm-forming *E. coli* O157:H7 strain did not produce cellulose as a component of the extracellular matrix. In light of the conflicting role of curli and cellulose in *E. coli* O157:H7 attachment to plant surfaces, we used two *E. coli* O157:H7 strains, which were isolated from two independent human disease outbreaks, to discern the role of curli and cellulose in attachment of this important food-borne pathotype of *E. coli* to spinach surfaces. We used isogenic mutants of *E. coli* O157:H7 strain EDL933 and 86-24 to determine the relative contributions of curli and cellulose in qualitative and quantitative bacterial adherence to spinach leaves.

## Methods

### Plant material

Organic spinach (*Emilia* cultivar) seeds were obtained from a commercial seed supplier (Seedway, Hall, NY). Approximately 300 seeds (2.84 g) were surface sterilized for 30 min with 1.0% sodium hypochlorite (5%; Sigma, St. Louis, MO), followed by five washes with sterile deionized water. To promote uniform and vigorous germination, seeds were primed in 200 mL of solution containing 30% (wt/vol) polyethylene glycol (PEG) 8000 (Sigma) for 72 h as recommended by Hart *et al.* (2006). After priming, PEG was removed from the seeds by washing, and seeds were planted in a "cone-tainer" (Stuewe & Sons, Inc., Corvallis, OR) filled with Miracle-Gro garden soil (Miracle-Gro, Marysville, OH) and containing a new glass fiber wad at the base (to prevent soil from passing out of the drainage holes). Germination and seedling growth were carried out in a growth chamber at 22°C and a relative humidity of 50–60% using a photoperiod consisting of 18 h of light (600  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 6 h of darkness. Spinach plants were irrigated as needed to maintain soil moisture and plant turgor.

### Bacterial strains and mutant construction

Shiga toxin-producing *E. coli* O157:H7 wild-type strains and their mutants (lacking the ability to produce curli or cellulose or both) used in the current study are listed in Table 1. *E. coli* O157:H7 strain EDL933 and its isogenic mutants have

been described in a previous study (Saldana *et al.*, 2009). The *csgA* and *csgD* mutants of *E. coli* O157:H7 strain 86-24 were constructed by using the bacteriophage  $\lambda$ -red-mediated recombination method as described previously (Datsenko and Wanner, 2000). Two successive transfers of cryo-preserved (-80°C) *E. coli* strains were made in Luria Bertani broth (LB; Neogen, Lansing, MI) at 37°C for 24 h. Streptomycin (100  $\mu\text{g/ml}$ ), kanamycin (50  $\mu\text{g/ml}$ ), ampicillin (100  $\mu\text{g/ml}$ ), and chloramphenicol (34  $\mu\text{g/ml}$ ) were added to LB as required for the growth of bacterial strains used in this study (Table 1).

### Congo red and calcofluor staining

To identify the optimal temperature for curli production, overnight cultures of two *E. coli* O157:H7 strains and their mutants were streaked on LB agar plates devoid of salt and supplemented with Congo Red (40  $\mu\text{g/ml}$ ; Sigma-Aldrich, St. Louis, MO) and Coomassie brilliant blue (20  $\mu\text{g/ml}$ ; Sigma-Aldrich) (Römling *et al.*, 2003), and incubated at 18°C, 20°C, 22°C, 28°C, and 37°C for 48 h. Bacteria-producing curli developed red colonies as a result of dye uptake. To detect cellulose production, bacteria were grown on LB plates without salt containing 200 mg/L calcofluor (Uhlich *et al.*, 2006) and incubated at 18°C and 22°C for 48 h with cellulose-producing colonies fluorescing under ultraviolet (UV) light.

### Inoculum preparation

Bacterial strains (Table 1) were grown in 10 mL of LB broth at 37°C and, when required, supplemented with antibiotics: kanamycin (50  $\mu\text{g/ml}$ ), streptomycin (100  $\mu\text{g/ml}$ ), chloramphenicol (30  $\mu\text{g/ml}$ ), and ampicillin (200  $\mu\text{g/ml}$ ). After 24 h of growth, 1 mL of bacterial culture was centrifuged at 5000  $\times g$  for 15 min. Pelleted cells were washed with phosphate-buffered saline (PBS) and resuspended in 40 mL of PBS. Spinach leaves of approximately the same size (approximately 2  $\times$  3 cm) were harvested from 5-week-old plants and then spot inoculated with 100  $\mu\text{L}$  of suspension (5 spots of 20  $\mu\text{L}$  of inoculums per leaf) to obtain 7 log colony forming units (CFU) of bacteria/leaf. Uninoculated leaves were used as controls.

### Adherence assays

At 0, 24, and 48 h after incubation, loosely and strongly attached *E. coli* O157:H7 populations were determined as

TABLE 1. BACTERIAL STRAINS AND PLASMIDS USED FOR THIS STUDY

Strain or plasmid	Relevant characteristics	Reference or source
86-24	<i>E. coli</i> (O157:H7), wild-type, curli <sup>+</sup> , cellulose <sup>+</sup> , Sm <sup>r</sup>	Griffin <i>et al.</i> , 1988
86-24 $\Delta$ <i>csgA</i>	<i>E. coli</i> (O157:H7), <i>csgA</i> mutant, curli <sup>-</sup> , cellulose <sup>+</sup> , Sm <sup>r</sup>	Unpublished (Vijay Sharma)
86-24 <i>csgD</i> <sup>c</sup>	<i>E. coli</i> (O157:H7), constitutive <i>csgD</i> mutant, curli <sup>+</sup> , cellulose <sup>+</sup> , Sm <sup>r</sup>	Unpublished (Vijay Sharma)
EDL933	<i>E. coli</i> (O157:H7), wild-type, curli <sup>+</sup> , cellulose <sup>+</sup>	Saldana <i>et al.</i> (2009)
EDL933 $\Delta$ <i>csgA</i>	<i>E. coli</i> (O157:H7), <i>csgA</i> mutant, curli <sup>-</sup> , cellulose <sup>+</sup> , Km <sup>r</sup>	Saldana <i>et al.</i> (2009)
EDL933 $\Delta$ <i>csgA</i> (pC <i>csgA</i> )	<i>E. coli</i> (O157:H7), restored <i>csgA</i> mutant, curli <sup>+</sup> , cellulose <sup>+</sup> , Ap <sup>r</sup>	Saldana <i>et al.</i> (2009)
EDL933 $\Delta$ <i>csgD</i>	<i>E. coli</i> (O157:H7), <i>csgD</i> mutant, curli <sup>-</sup> , cellulose <sup>+</sup> , Km <sup>r</sup>	Saldana <i>et al.</i> (2009)
EDL933 $\Delta$ <i>csgA</i> / $\Delta$ <i>bcsA</i>	<i>E. coli</i> (O157:H7), Double <i>csgA</i> / <i>bcsA</i> mutant, curli <sup>-</sup> , cellulose <sup>-</sup> , Km <sup>r</sup> , Cm <sup>r</sup>	Saldana <i>et al.</i> (2009)
EDL933 $\Delta$ <i>bcsA</i>	<i>E. coli</i> (O157:H7), <i>bcsA</i> mutant, curli <sup>+</sup> , cellulose <sup>-</sup> , Cm <sup>r</sup>	Saldana <i>et al.</i> (2009)

curli<sup>+</sup>, curli-expressing strain; curli<sup>-</sup>, curli-deficient strain; cellulose<sup>+</sup>, cellulose-expressing strain; cellulose<sup>-</sup>, cellulose-deficient strain; Sm<sup>r</sup>, streptomycin resistant; Ap<sup>r</sup>, ampicillin resistant; Km<sup>r</sup>, kanamycin resistant; Cm<sup>r</sup>, chloramphenicol resistant.

described by Patel *et al.* (2011). Briefly, inoculated spinach leaves were washed in sterile PBS to remove unattached bacteria; leaves were transferred to 50-mL conical tubes filled with 20 mL of sterile PBS containing 0.05% Tween 20 (PBS-TW) and vortexed for 2 min to dislodge loosely attached bacteria. After vortexing, leaves were transferred to new 50-mL conical tubes containing 20 mL PBS-TW and homogenized using a Polytron® 2100 homogenizer (Kinematica, Littau-Lucerne, Switzerland) to determine populations of strongly attached bacteria. PBS (loose attachment) and homogenate (strong attachment) were spiral plated on SMAC agar supplemented when required with a corresponding antibiotic (Table 1). After overnight incubation at 37°C, presumptive *E. coli* O157:H7 colonies were counted using automated colony counter (ProtoCOL; Microbiology International Inc., Frederick, MD). Randomly selected colonies were confirmed for *E. coli* O157 lipopolysaccharide (LPS) antigens by latex agglutination assay (Dryspot assay; Oxoid Ltd., Cambridge, UK). The relative attachment strength ( $S_R$ ) was determined as earlier described by Dickson and Koohmaraie (1989) and represents the ratio of the strongly attached bacterial cells to the total population of bacteria associated with the produce surface [ $S_R = (\text{strongly attached bacteria}) / (\text{strongly attached} + \text{loosely associated bacteria})$ ].

#### LSCM analysis

At 24 and 48 hpi (hours post inoculation), leaves were washed three times in PBS to remove unattached bacteria and fixed in  $-40^\circ\text{C}$  methanol for 5 min, followed by three washes with PBS and incubation for 30 min in PBS containing 2% non-fat dry milk to block non-specific immunoglobulin binding in subsequent steps. After blocking, the samples were incubated with rabbit anti-curli polyclonal antibodies (1:1000 dilution) (Saldana *et al.*, 2009) for 2 h at 37°C, followed by 2-h incubation with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit immunoglobulin G (IgG, 1:50 dilution; Sigma). All antibodies were diluted in PBS-Tween 20 (PBS-TW), and removed after each step by three washes with PBS-TW. A Zeiss 710 LSCM system with 63 $\times$  and 100 $\times$ 1.4 NA oil immersion Plan Apochromatic objectives was utilized in immunofluorescence analysis. Differential interference contrast (DIC) and confocal fluorescence images were acquired simultaneously. A photomultiplier tube captured the light emitted from a 488-nm argon laser with a 3.7- $\mu\text{m}$  pin hole passing through an MBS 488 filter with limits set between 492 and 543 nm for detection of fluorescein and between 647 and 721 nm for detection of autofluorescence from chloroplasts. Zeiss Zen 2008 software was used to obtain the images with 512 $\times$ 512 pixel resolution.

#### Statistical analysis

A randomized complete block design was used with three replicates per treatment. The populations of loosely and strongly attached *E. coli* O157:H7 obtained at each sampling period were converted to log CFU/g. The data obtained from three replicates were analyzed by a two-way analysis of variance (ANOVA) using Proc Mixed (SAS 8.2; SAS, Cary, NC) for interaction effects of the strain and sampling period. The results were considered statistically significant at  $p < 0.01$ .

## Results

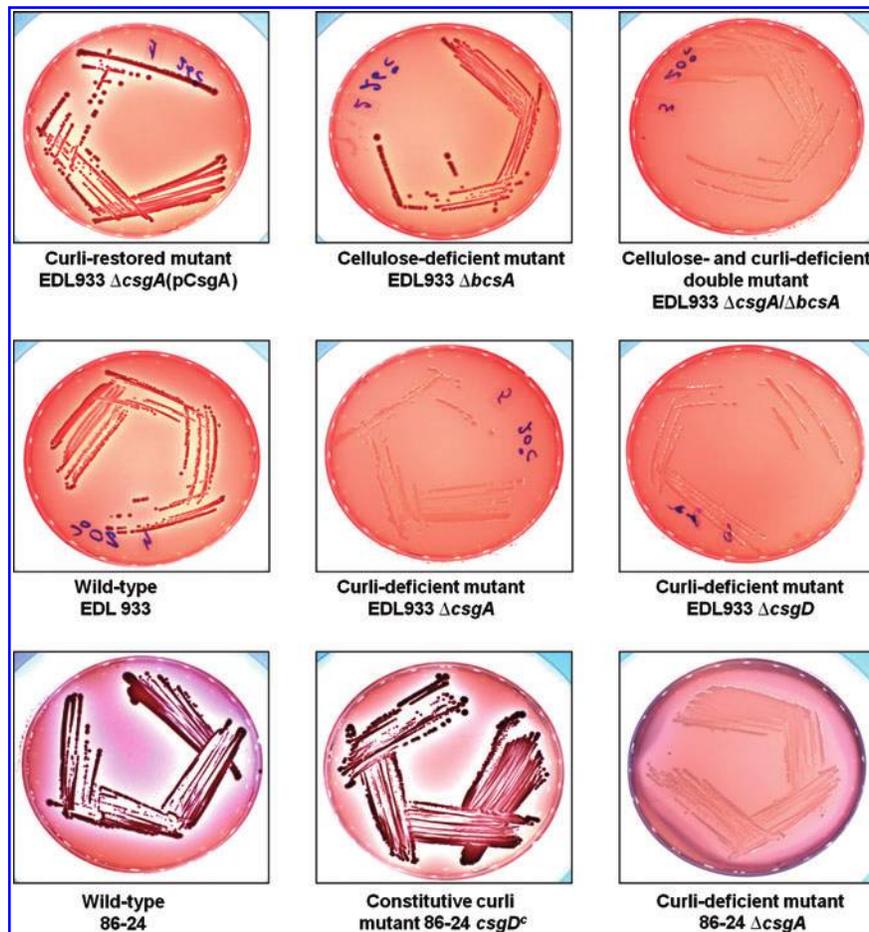
The highest levels of curli production were observed after incubation at 22°C. *E. coli* O157:H7 strains EDL933 $\Delta\text{csgA}$ , EDL933 $\Delta\text{csgD}$ , EDL933 $\Delta\text{csgA}/\Delta\text{bcsA}$ , and 86-24 $\Delta\text{csgA}$  mutants deficient in curli or curli and cellulose production did not develop red phenotype on Congo red media at any incubation temperature used in the study. Among the curli-producing and Congo red-binding strains, the strongest level of curli expression was observed for the wild-type *E. coli* O157:H7 strain 86-24 and its constitutive curli mutant 86-24  $\text{csgD}^c$  (Fig. 1). All strains, except EDL933 $\Delta\text{bcsA}$  and EDL933 $\Delta\text{bcsA}/\Delta\text{csgA}$ , produced cellulose (data not shown). Since the greatest level of curli expression was observed at 22°C (also accompanied by cellulose biosynthesis), this temperature was used in further analysis of bacterial attachment to spinach leaves.

Immediately after inoculation (Time 0), numbers of loosely attached bacteria were not significantly different among the *E. coli* O157:H7 strains, ranging from 4.2 to 4.5 log CFU/leaf (data not shown). After 24-h incubation, numbers of loosely attached bacteria were similar to those observed at time 0, with the exception of EDL933 $\Delta\text{csgD}$ , which showed significantly ( $p > 0.01$ ) lower recovery (3.7 log CFU/leaf). After 48-h incubation, numbers of loosely attached bacteria on the spinach leaves were in the range of 4.5 to 4.9 log CFU/leaf and did not vary significantly ( $p > 0.01$ ) among mutants and parental strains. Recovery of loosely attached bacteria indicated that, after undergoing initial die-off, overall curli- and cellulose-impaired strains maintained relatively stable populations of loosely attached cells similar to those of curli- and cellulose-expressing genotypes; nevertheless curli-negative EDL933 $\text{csgD}$  mutant (deficient in CsgD transcriptional activator) showed the least attachment to leaf surface at 24 h post inoculation.

At time 0, populations of strongly attached bacteria recovered from the spinach leaves ranged from 2.2 to 3.3 log CFU/leaf (0 hpi; Fig. 2). Curli-expressing *E. coli* O157:H7 strains developed stronger association with leaf surface with time, as indicated by the presence of significantly higher numbers of strongly attached cells (4.1–4.8 log CFU/leaf) by 24 h of incubation (Fig. 2). Curli-deficient mutants, however, failed to attach at higher numbers after 24 h. Strongly attached populations of *E. coli* O157:H7 strains EDL933 $\Delta\text{csgA}$  (3.2 log CFU/g) and EDL933 $\Delta\text{csgA}/\Delta\text{bcsA}$  (3.6 log CFU/g) were similar to their corresponding populations at time 0. Populations of strongly attached curli-impaired mutants EDL933 $\Delta\text{csgD}$  and 86-24 $\Delta\text{csgA}$  decreased within 24 h to 3.0 and 2.4 log CFU/leaf, respectively. After 48 h, numbers of curli-deficient bacteria strongly attaching to spinach have increased.

At 0 hpi, the attachment strength ( $S_R$ ) of *E. coli* O157:H7 to spinach was of low and variable magnitude (0 hpi in Fig. 3). With time, attachment strength significantly increased for the curli-expressing strains; at 24 h, the difference in  $S_R$  values between curli-producing and curli-deficient strains ranged from 1.5 to 200-fold (24 hpi in Fig. 3). At 48 h, the differences in the attachment strengths remained significant among curli-competent and curli-impaired *E. coli* O157:H7 strains (48 hpi in Fig. 3).

LSCM analysis of inoculated spinach leaves revealed that bacteria firmly attached on leaf surface were surrounded by



**FIG. 1.** Phenotypic presentation of *Escherichia coli* O157:H7 curli variants grown on Luria Bertani broth (LB; Neogen, Lansing, MI)/no-salt medium supplemented with Congo Red at 22°C for 48 h. The intensity of colony pigmentation as a result of Congo Red binding indicates different level of curli production. Color images available online at [www.liebertonline.com/fpd](http://www.liebertonline.com/fpd)

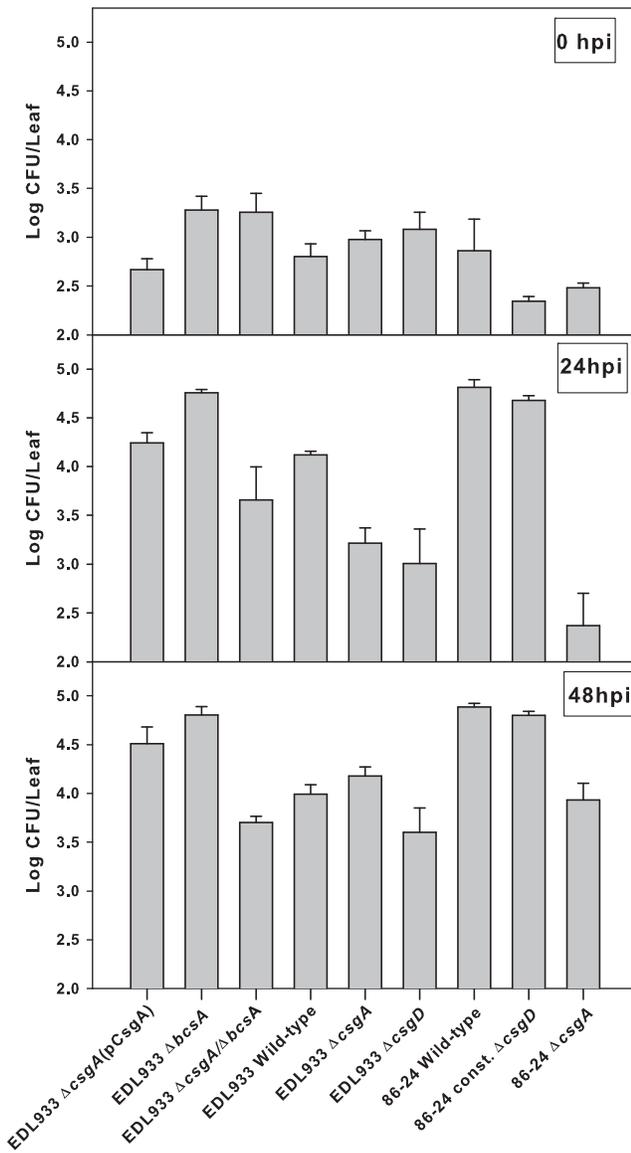
an extracellular matrix as recognized by rabbit anti-curli antibodies. Figure 4 shows leaf surface colonized by *E. coli* O157:H7 constitutive mutant 86-24*csgD<sup>c</sup>* (Fig. 4A–C) and wild-type parental strain 86-24 (Fig. 4D–J), both intensely immunostained for curli. Curli-impaired EDL933*ΔcsgD* and 86-24*ΔcsgA* mutant strains, which attached at a significantly lower numbers ( $p < 0.01$ ) on spinach at 24 hpi, were not recognized by anti-curli polyclonal antibodies (Fig. 5). Immunofluorescence analysis of inoculated spinach leaves did not detect curli expression by EDL933*ΔcsgA/ΔbcsA*, EDL933*ΔcsgA*, EDL933*ΔcsgD*, or 86-24*ΔcsgA*, at 48 hpi (data not shown).

## Discussion

The present study was conducted to address the potential use of curli and cellulose by *E. coli* O157:H7, an important foodborne human pathogen, to colonize and persist on fresh produce. Specifically, the effect of curli and cellulose expression on *E. coli* O157:H7 attachment to spinach leaves was evaluated. Earlier research has supported the involvement of these cellular appendices in *E. coli* O157:H7 attachment to leafy greens (Torres *et al.*, 2005; Jeter and Matthyse, 2005; Matthyse *et al.*, 2008; Patel *et al.*, 2010; Saldana *et al.*, 2011). On

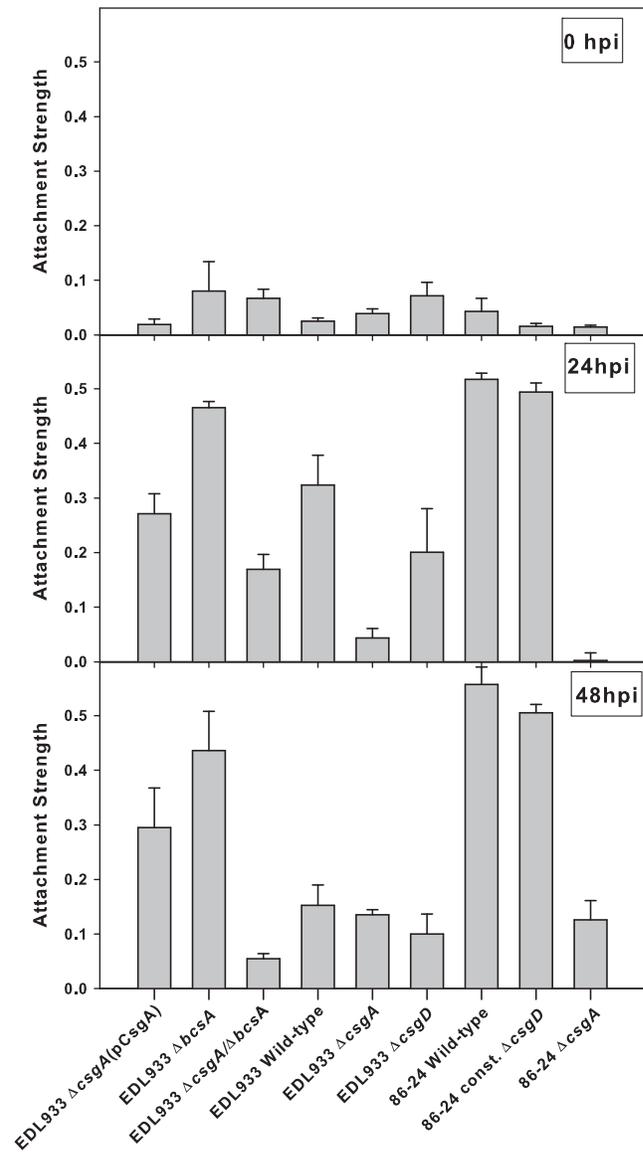
the other hand, there are studies showing no role for curli and cellulose in colonization and persistence on plant surfaces (Uhlich *et al.*, 2006; Boyer *et al.*, 2007). Production of curli and cellulose varied among *E. coli* strains and was highly responsive to external stimuli (Carter *et al.*, 2011) which could have explained differences in results of these studies. To minimize the strain effects in evaluating the role of curli and cellulose expression in *E. coli* O157:H7 attachment to spinach leaves, two lineages of well-studied enterohemorrhagic *E. coli* O157:H7 strains, EDL933 and 86-24, and their isogenic mutants were included in the current study. Also, to reduce the number of variables while assessing the role of cellular appendices in bacterial colonization of leaf surface, inoculated spinach was incubated at 22°C, an optimal temperature for curli and cellulose production by these strains. Further studies are needed to determine the role of curli and cellulose in bacterial adherence to and biofilm formation on produce surfaces at 4°C in commercial storage settings.

Experiments on the recovery of loosely attached bacteria to spinach leaves showed that the populations of curli- and cellulose-positive strains were comparable to those of curli- and cellulose-negative mutants at all sampling periods, suggesting that production of curli and cellulose or lack thereof by strains EDL933 and 86-24 had no effect on loose attachment



**FIG. 2.** Populations of strongly attached *Escherichia coli* O157:H7 and its curli and cellulose mutants to spinach leaf incubated at 22°C. Counts (Log colony forming unit [CFU]/leaf) obtained from Sorbitol MacConkey (SMAC) agar containing antibiotics (as needed). Error bars are the standard deviations from three replications. hpi, hours post inoculation.

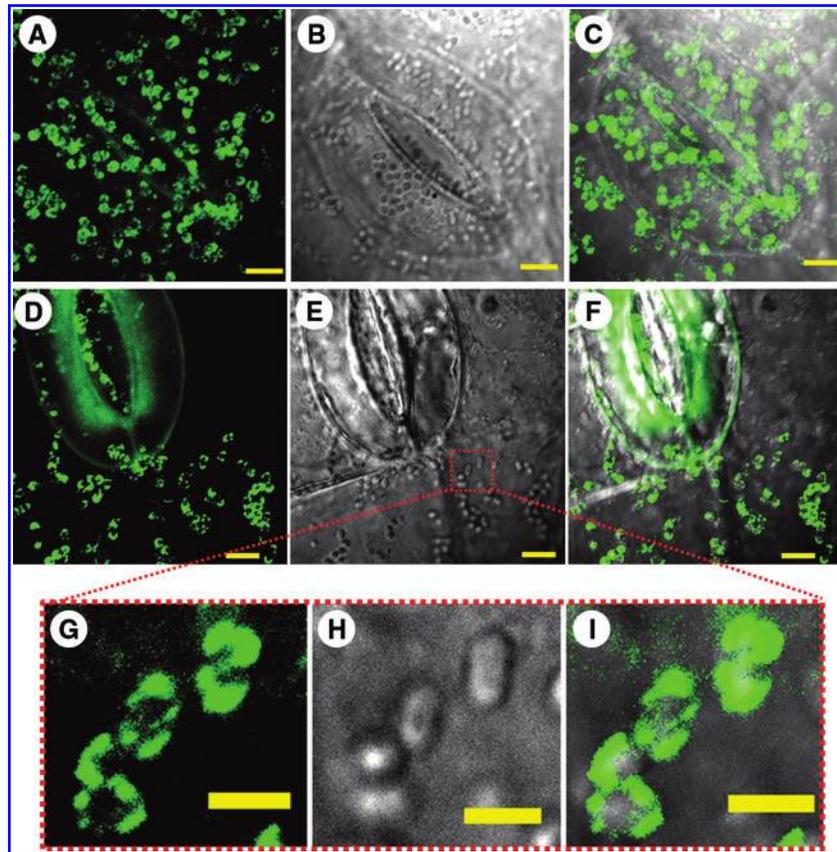
of *E. coli* O157:H7 to spinach. Recovery of strongly attached curli-deficient mutants, EDL933 $\Delta$ csgA/ $\Delta$ bcsA, EDL933 $\Delta$ csgA, EDL933 $\Delta$ csgD, and 86-24 $\Delta$ csgA, were significantly lower than the recovery of strongly attached curli-expressing mutants and wild-type parental strains. In a recent study, Saldana *et al.* (2011) reported that, among known adherence factors (such as *E. coli* common pilus, T3SS, flagella), *E. coli* O157:H7 strain EDL933 also utilizes curli to colonize baby spinach leaves. In our study, curli were critical in strong attachment to spinach, whereas loose attachment to spinach was not affected by curli expression. The differences could be due to the different methodology used in quantitative assessment of bacterial at-



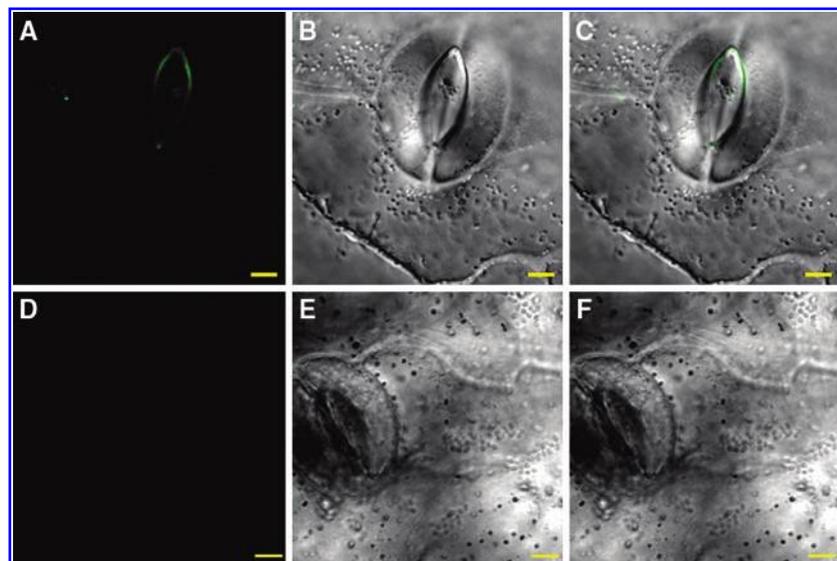
**FIG. 3.** Attachment strength of *Escherichia coli* O157:H7 and its curli and cellulose mutants to spinach leaf incubated at 22°C. The relative attachment strength ( $S_R$ ) values were calculated as the ratio of strongly attached cells/(strongly+loosely attached cells). Error bars indicate the standard deviations from three replications. hpi, hours post inoculation.

tachment. Unlike the work of Saldana *et al.* (2011), this study differentiated bacterial attachment to spinach leaves in loose and strong attachment.

In our study, lack of cellulose expression by EDL933 $\Delta$ bcsA did not affect its ability to develop firm binding to leaf surface. Integral to that, deletion of the *bcsA* gene in a curli/cellulose-impaired mutant (EDL933 $\Delta$ csgA/ $\Delta$ bcsA) did not reduce its binding capacity to spinach as compared to non-curliated EDL933 $\Delta$ csgD and 86-24 $\Delta$ csgA. Our results are in agreement with Uhlich *et al.* (2006), who reported that cellulose biosynthesis was not necessary in *E. coli* O157:H7 attachment and biofilm formation on inanimate surfaces.



**FIG. 4.** Laser scanning confocal microscopy (LSCM) images of a constitutive curli mutant 86-24 *csgD<sup>c</sup>* (A–C) and wild-type strain 86-24 (D–I) attached to the surface of the spinach leaf. Green fluorescence in single-channel fluorescence images (left column) indicates the presence of curly recognized by anti-curli polyclonal antibodies. Guard cells also slightly fluoresce green as a result of cell wall autofluorescence. Differential interference contrast (DIC) images (central column) illustrate bacteria cells adhering to the guard and epidermal cells and within stomata opening. Right column shows overlays of the DIC and fluorescence images. Panel G–I presents a magnified view of bacteria producing extracellular matrix intensively immunostained with anti-curli antibodies. Scale bar = 5  $\mu\text{m}$  (A–F), 2  $\mu\text{m}$  (G–I). Color images available online at [www.liebertonline.com/fpd](http://www.liebertonline.com/fpd)



**FIG. 5.** Laser scanning confocal microscopy (LSCM) images of *Escherichia coli* O157:H7 curli-deficient mutants EDL933 $\Delta$ *csgD* (A–C) and 86-24 $\Delta$ *csgA* (D–F) on the surface of the spinach leaf. No green fluorescence was detected on single-channel fluorescence images (left column) except moderate autofluorescence of the guard cell wall. Differential interference contrast (DIC) images (middle column) illustrate bacteria cells adhering to the epidermal cells in vicinity of stomata. Right column shows overlays of the DIC and fluorescence images. No immunoreactivity for curli was detected. Scale bar = 5  $\mu\text{m}$ . Color images available online at [www.liebertonline.com/fpd](http://www.liebertonline.com/fpd)

Attachment strength ( $S_R$ ) correlated with bacterial ability to produce curli. Both wild-type strains and their curled mutants had significantly higher  $S_R$  values as compared to curli-deficient strains. Notably, the strength of attachment of *E. coli* O157:H7 to Romaine lettuce surface also positively correlated with curli expression (Patel *et al.*, 2011). The differences in the attachment strength between curli negative and curli-producing strains increased with incubation time, which were likely due to the gradual increases in curli biosynthesis in the curli-positive strains resulting in stronger adherence to leaf surfaces. Our data are in agreement with previous study showing a significant increase in the attachment strength of curli-expressing *Salmonella* serovars to produce surfaces within a 24-h time interval (Patel *et al.*, 2010). The attachment strength of cellulose-deficient mutant strain EDL933 $\Delta$ *bcsA* was not significantly different from the attachment strength of cellulose-expressing strains, indicating that this polysaccharide is not critical for *E. coli* O157:H7 attachment to spinach. This observation disagrees with an earlier report showing role of cellulose in bacterial colonization of plant surfaces (Barak *et al.*, 2007). Differences in current results and those from the Barak *et al.* (2007) study may be due to different mechanisms of attachment between *Salmonella* and *E. coli* O157:H7.

In conclusion, the present study shows that loose attachment of *E. coli* O157:H7 to spinach leaves is not affected by extracellular appendices such as curli and cellulose. However, strong attachment of *E. coli* to plant surface during the first 24 h after inoculation is greatly influenced by its ability to produce curli fimbriae. A sharp increase in populations of the curli-expressing *E. coli* O157:H7 within first 24 h after inoculation, together with LSCM observation of curli-composed extracellular matrix on leaf surfaces, clearly indicate that curli are critical in strong attachment to fresh produce. Cellulose production is not required for strong attachment to the spinach leaf by *E. coli* O157:H7 strains used in this study. A moderate increase in populations of strongly attached cells of both curli-positive and curli-deficient *E. coli* strains after 48 h suggests that, in addition to curli, some other bacterial adherence factors (e.g., flagella [Xicohtencatl-Cortes *et al.*, 2009] or the type 3 secretion system [Saldana *et al.*, 2011]) become involved at subsequent stages in persistent association with the spinach leaf.

### Disclosure Statement

No competing financial interests exist.

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