Running Title: *E. coli* O104:H4 AAF/I

**Aggregative Adherence Fimbriae I (AAF/I) Mediate Colonization of Fresh Produce and
Abiotic Surface by Shiga Toxigenic Enteroaggregative *Escherichia coli* O104:H4**

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ABSTRACT

The Shigatoxigenic *Escherichia coli* O104:H4 isolated during the 2011 European outbreak expresses shiga toxin 2a and possess virulence genes associated with the enteroaggregative *E. coli* (EAEC) pathotype. It produces plasmid encoded aggregative adherence fimbriae I (AAF/I) which mediate cell aggregation and biofilm formation in human intestine and promote Shiga-toxin adsorption, but it is not clear whether the AAF/I fimbriae are involved in the colonization and biofilm formation on food and environmental matrices such as the surface of fresh produce. We deleted the gene encoding for the AAF/I fimbriae main subunit (AggA) from an outbreak associated *E. coli* O104:H4 strain, and evaluated the role of AAF/I fimbriae in the adherence and colonization of *E. coli* O104:H4 to spinach and abiotic surfaces. The deletion of *aggA* did not affect the adherence of *E. coli* O104:H4 to these surfaces. However, it severely diminished the colonization and biofilm formation of *E. coli* O104:H4 on these surfaces. Strong aggregation and biofilm formation on spinach and abiotic surfaces were observed with the wild type strain but not the isogenic *aggA* deletion mutant, suggesting that AAF/I fimbriae play a crucial role in persistence of O104:H4 cells outside of the intestines of host species, such as on the surface of fresh produce.

Key words: Enteroaggregative *E. coli*, *E. coli* O104:H4, aggregative adherence fimbriae, fresh produce, aggregation, colonization, biofilms

INTRODUCTION

In 2011, a major foodborne outbreak of diarrhea and hemolytic uremic syndrome (HUS) in Germany and other European countries was associated with consumption of contaminated sprouts (Buchholz et al., 2011). This large scale outbreak was caused by a novel strain that closely resembles the enteroaggregative *Escherichia coli* (EAEC) serotype O104:H4, but it also harbors a prophage encoding Shiga-toxin 2a (Stx2a), which is the characteristic of **shiga toxin producing *E. coli* (STEC)** (Bielaszewska et al., 2011; Mellmann et al., 2011; Rasko et al., 2011). The novel combination of virulence factors in one strain lead to the largest HUS outbreak in record, with nearly 4000 infected persons resulted in more than 800 confirmed cases of HUS and 54 deaths.

The *E. coli* serotype O104:H4 **outbreak strain** contains three plasmids (1.5 kb pG-EA11, 7.4 kb pAA-EA11, and 8.9 kb pESBL-EA11) (Ahmed et al., 2012; Brzuszkiewicz et al., 2011; Mellmann et al., 2011; Rasko et al., 2011; Rohde et al., 2011). The pAA-EA11 plasmid carries several EAEC-virulence loci (Ahmed et al., 2012; Brzuszkiewicz et al., 2011), including the *aggABCD* adherence fimbrial cluster, which encodes the aggregative adherence fimbriae I (AAF/I). AggA, the main subunits, assemble into linear polymers and form the fimbrial shaft (Berry et al., 2014).

EAEC **attached to the surface of various epithelial cell lines displays highly ordered aggregative adhesion pattern (Nataro, 2005)**. Aggregative fimbriae AAF/I and AAF/II have been implicated in the formation of the “stacked brick” structure of EAEC cells (Bielaszewska et al., 2011). It was hypothesized (Beutin and Martin, 2012; Bielaszewska et al., 2011; Nataro, 2005) that the AAF/I-mediated adherence of *E. coli* O104:H4 to human intestinal epithelial cells facilitates absorption of the **toxin** Stx2a, which is directly related the pathogenesis of HUS.

Even though the process of AAF/I fimbriae-mediated biofilm formation on the surface of intestinal epithelia is a well recognized phenomenon (Torres et al., 2012), little is known about the role of these surface structures in the attachment and persistence of *E. coli* O104:H4 cells on fresh produce. In this study we evaluated the role of aggregative adherence fimbriae I in bacterial adherence and colonization of fresh produce and abiotic surfaces, and showed that the deletion of the gene coding for the AAF/I major subunit did not hinder the ability of O104:H4 cells to attach to fresh produce or abiotic surfaces, but it significantly reduced the ability of this strain to form multilayered aggregates, suggesting AAF/I play an important role in the process of surface colonization and biofilm formation outside human or animal hosts.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* O104:H4 strain TW16133 was obtained from Michigan State University EHEC Stock Center. It was previously isolated from patient infected during travel to Germany and was confirmed identical to the outbreak strain by molecular typing procedures (Safadi et al, 2012). Tryptic soy broth (TSB) or Luria-Bertani broth (LB), and tryptic soy agar (TSA) or LB agar (Becton Dickinson, Franklin Lakes, NJ, and Acumedia, Neogen, Lansing, MI) are used for routine growth of the bacterial strains. When required, antibiotics were added to the media at the following concentrations: kanamycin (Kan, 20 and 34 µg/ml), ampicillin (Amp, 100 µg/ml), gentamicin (Gen, 10 µg/ml), chloramphenicol (Cm, 20 µg/ml) (Sigma, Saint Louis, MO).

86 **Recombinant DNA techniques, construction of isogenic mutants.** The λ Red recombination
87 technology (Datsenko and Wanner, 2000) was used for in-frame deletion of the *aggA* coding
88 sequence. Plasmid pKD78 (Yale *E. coli* Genetic Stock Center, New Heaven, CT), which is a
89 chloramphenicol resistant derivative of the original ampicillin resistant pKD46 (Datsenko and
90 Wanner, unpublished), was transformed into *E. coli* O104:H4 TW16133 (Ahmed et al., 2012) by
91 electroporation using a Gene Pulser Transfection Apparatus with a Pulse Controller (BioRad,
92 Hercules, CA). A linear mutagenesis DNA product with target sequences flanking kanamycin
93 resistance gene (*kan*) was generated by PCR using pKD4 as template and oligonucleotides AAF-
94 5 and AAF-3 (Table 1) designed as described by Datsenko and Wanner (2000) as primers. While
95 the transformation of TW16133/pKD78 with linear mutagenesis DNA and the selection of
96 recombinant colonies in general followed the original protocol of Datsenko and Wanner, limited
97 optimization was also conducted for mutagenesis in *E. coli* O104:H4, in reference to previous
98 modifications for mutagenesis in *E. coli* O157:H7 (Serra-Moreno et al., 2006). Briefly,
99 TW16133/pKD78 was grown in 25 mL LB with 20 μ g/ml chloramphenicol at 30°C to OD₆₀₀ $\approx$
100 0.6. The expression of λ Red recombinase was induced with 100 mM L-arabinose approximately
101 1 h prior to cell harvesting. Induced TW16133/pKD78 cells were made electro-competent by
102 concentrating 100-fold and washing three times with ice-cold MilliQ water. Gel purified linear
103 mutagenesis PCR product (10-100 ng) was electroporated into 100 μ l competent cells at 1.8 kV,
104 100 Ohms resistance and 25 μ F capacitance. Shocked cells were incubated for 3 h at 37°C in 1
105 ml SOC broth (Becton Dickinson), and then spread onto LB agar with 20 μ g/ml kanamycin. The
106 elimination of pKD78 in the recombinant was confirmed by the sensitivity to chloramphenicol,
107 and the deletion of the *aggA* was verified with PCR, using oligonucleotide CTR-3, which anneals

to a sequence internal to the *kan* gene, and oligonucleotide CTR-5, which anneals to a sequence upstream of the deleted *aggA* coding sequence (Figure 1).

The gentamicin-resistant pGFP-Gen and pmCherry-Gen plasmids encoding for green and red fluorescence, respectively, were constructed by replacing the ampicillin resistance gene with gentamicin resistance gene from pFastBac1 in pGFP and pmCherry-C1 plasmids (Life Technologies, Carlsbad, CA), as previously described (Nagy et al., 2015). Each of these two plasmids were transformed into both the wild type and the mutant strains to facilitate differential observation of the strains using fluorescent microscopes.

Cell growth assessment. Individual bacterial strains were grown in 200 µl TSB in 96-well microplates at 37 °C with shaking for 24 h. A Synergy 4 Hybrid microplate reader controlled by Gen5 Software (BioTek Instruments, Winooski, VT) was used for microplate incubation and for obtaining bacterial growth kinetics information by measuring OD₆₀₀ at 10 min intervals during the incubation. Data were analyzed using OriginPro 7.5 (OriginLab Corporations, Northampton, MA). Optical doubling rates were calculated based on the slope of the linear phase of the growth curves. Experiment was repeated 3 times.

The wild type and the *ΔaggA* mutant strains were also grown together to assess the effect of the mutation on competitiveness of growth. Overnight cultures of GFP labeled wild type and mCherry labeled mutant strain were mixed at 1:1 ratio and diluted (1:1000) in LB broth supplemented with gentamycin (10 mg/L). The growth at 37 °C was monitored for 24 h by measuring OD₆₀₀. Samples were also taken at 0, 4, 8, 12, and 24 h and examined using EVOS fluorescence Auto Imaging System (Thermo Fisher Scientific, Waltham, MA) to determine the ratio of green and red fluorescent cells in the co-culture. Green and red fluorescent cells in 10

randomly selected frames were counted using ImageJ (NIH, Bethesda, MD). Competitive growth index (CGI) for the mutant was calculated as the ratio of *ΔaggA* mutant (red) and wild type (green) cells at each sampling point, with normalization so that the initial CGI at inoculation was equal to 1.00.

Biofilm formation assessment. The wild type and *ΔaggA* mutant strains were grown statically in 3 ml of TSB for 24 h at 25 °C. After removal of cell suspension, cells attached to the sides of the glass tubes were gently rinsed twice with 5 ml of PBS and air dried, followed by staining with 0.25% Coomassie Blue R-250 (50% methanol, 10% acetic acid and 40 % distilled water solution) for 5 minutes. After staining the tubes were rinsed with distilled water. After air drying, the stained pellicle ring formed at the liquid-air interface was photographed.

Total biomass of wild type and *ΔaggA* mutant strains was quantified using crystal violet staining assay as previously described by Stepanovic et al. (2000) **after growth in 10% TSB.** Three non-inoculated but otherwise identically treated wells were used as negative controls. The cut-off OD (OD_c) was defined as three standard deviations above the mean OD of the negative control values. The biofilm formation of each strain was classified as “Negative” (OD/OD_c ≤ 1), “Weak” (1 < OD/OD_c ≤ 2), “Moderate” (2 < OD/OD_c ≤ 4), or “Strong” (OD/OD_c > 4) as previously described (Liu et al., 2013; Stepanovic et al., 2000).

Adherence to spinach and romaine lettuce. Baby spinach and romaine lettuce purchased from a local store were used for inoculation. The leaves of produce (25 g) were inoculated by submerging for 10 minutes in a PBS solution containing approximately 10⁶ cells of TW16133/pGFP-Gen and TW16133 *ΔaggA*/pmCherry-Gen mixed at 1:1 ratio. After draining,

the leaves were stored at 4 °C overnight to facilitate closer attachment of bacterial cells to the produce surface without significant cell proliferation or biofilm formation. To enumerate the attached WT and mutant cells, 5 g portions of inoculated leaves were either washed twice in 200 ml distilled water (DW), or washed one time in 200 ml of 50 mg/L of sodium hypochlorite (pH 6.5 acidified using citric acid) followed by a 200 ml DW rinsing. This was done to allow the assessment of competitive adherence of the WT and the mutant strains under different washing stringencies. Portions of unwashed and washed leaves were mixed with 5 ml of PBS in 30 ml homogenization sample tubes with 2.8 mm ceramic beads (Omni International, Kennesaw, GA) and were homogenized in Omni Bead Ruptor 24 Homogenizer for two times at 30 s at 4 m/s speed. The inoculums and the samples after homogenization were diluted and spiral plated on LB agar plates containing 100 µg/ml ampicillin using Eddy Jet 2 spiral plater (Thermo Fisher Scientific, Pittsburgh, PA). The ratio of TW16133/pGFP and TW16133 *ΔaggA*/pmCherry colonies was determined by counting the green and red colonies on a UV transilluminator (UVP, Upland, CA).

Colonization of spinach and abiotic surface. To evaluate the effect of AAF/I fimbriae on the colonization of O104:H4 on produce surface, intact spinach leaves were inoculated by submerging in a cocktail of GFP labeled WT and mCherry labeled *ΔaggA* mutant prepared as above in 10% TSB. The spinach leaves and the bacterial suspension were incubated at 25 °C for 10 min, 4 h, and 24 h to allow cell attachment, proliferation, or biofilm formation. After incubation, spinach leaves were rinsed with PBS and examined by confocal laser scanning microscopy (CLSM). For the colonization on abiotic surface, glass bottomed Petri-dishes (Matech, Westlake Village, CA) were used in place of spinach leaves.

Confocal laser scanning microscopy (CLSM). Zeiss LSM710 confocal laser scanning microscopy (CLSM) system (Thornwood, NY) was used to obtain images. The images were observed using a Zeiss Axio Observer inverted microscope with 63x 1.4 NA Plan-Apochromat oil immersion objective. A 488 nm Argon laser and a 561 nm diode laser with a pin hole of 45µm passing through a MBS 488/561 beam splitter filter with limits set between 490-535 nm for GFP and 570-640 nm for mCherry. The Zeiss Zen 2012 (Thornwood, NY) 64-bit software was used to obtain 10-20 z-stack images with 1 µm spacing. Z-stack images were used to produce 3D renderings and to develop 2D maximum intensity projections. Data were analyzed using OriginPro 7.5.

Statistical analysis. Statistical analysis of the data for the bacterial enumeration was performed using GraphPad software (GraphPad Software Inc., La Jolla, CA). The statistical significance of the differences in the sample means of the mutant and wild-type strain was calculated using the unpaired Student's *t* test. Results were considered significant at a *P* value of < 0.05.

RESULTS

Generation of isogenic *ΔaggA* mutant and transformation with plasmids expressing fluorescent proteins. EAEC adherence to the human intestinal mucosa is mediated by aggregative adherence fimbriae AAF/I, which promotes the formation of multilayer biofilms. To gain better insight into the role of AAF/I in cellular attachment, we generated knockout null mutant in enteroaggregative *E. coli* O104:H4 TW16133 (Safadi et al., 2012) using λ Red recombinase mediated mutagenesis protocols (Datsenko and Wanner, 2000; Serra-Moreno et al.,

200 2006). Figure 1A schematically presents the construction of the isogenic deletion mutant,
201 showing the annealing positions of the oligonucleotide primers used for generating and
202 confirming of the mutant.

203 Functional kanamycin resistance gene (*kan*) was expressed in the isogenic mutant strain,
204 indicating successful replacement of the target gene (*aggA*) with *kan*. The deletion of the *aggA*
205 gene was confirmed by PCR reaction, using a forward primer specific to a sequence upstream of
206 the deleted *aggA* gene (CTR-5), and a reverse primer specific to a sequence in the knocked-in
207 *kan* gene (CTR-3). The size of the PCR fragment (500 bp) encompassing the deletion junctions
208 was consistent with those expected for the resultant mutant, confirming the successful deletion of
209 the *aggA* gene and insertion of the *kan* gene in place (Figure 1B).

210 Both the wild type and the deletion mutants were transformed with fluorescent protein
211 expressing plasmids (WT: pGFP-Gen expressing green and mutant: pmCherry-Gen expressing
212 red fluorescent proteins). These plasmids were expected to be stably maintained in these strains
213 for the duration of the ensuing experiments (24 h), as judged by the fluorescence of colonies
214 after consecutive routine streakings (>5) on non-selective plates.

215
216 **Growth comparison of wild type and isogenic *ΔaggA* mutant strains.** Deleting plasmid-
217 encoded proteins of the bacterial cells might influence the growth characteristics of the strain. In
218 this case, no difference was observed between the wild type and the mutant strain, as well as
219 their respective plasmids transformants, for colony morphology including shape, size and
220 appearance when growing on LB agar plates. We compared the growth kinetics of the wild type
221 *E. coli* and the mutant strains in LB broth. As shown in Figure 2A, the growth kinetics of the

deletion mutants was comparable to that of the WT strain, with nearly identical optical doubling times (~66 min).

The WT and the $\Delta aggA$ mutant were also grown competitively. GFP-labeled wild type and the mCherry-labeled $\Delta aggA$ mutant were grown together and the competitive growth index was determined by comparing the ratio of WT (green) and $\Delta aggA$ mutant (red) cells during the course of growth (Figure 2B). The growth of both the WT and the mutant was similar, with competitive growth index fluctuating between 0.76 ± 0.31 (24 h growth) and 1.19 ± 0.46 (12 h growth). The relative constant CGI indicated that the deletion of *aggA* did not significantly alter the growth characteristics in *E. coli* O104:H4

Effect of O104:H4 AAF/I fimbriae on biofilm formation. Biofilm formation by the wild type and the mutant strains were initially assessed by crystal violet staining of the pellicles at aerial-liquid interphase after incubation for 24 h at 25 °C (Figure 3A). It was clear that while the wild type strain formed a thick pellicle ring of biofilm at the interface, as well as on the sides of the immersed part of the tubes, the $\Delta aggA$ mutant's ability of biofilm formation as judged by the pellicle formation was severely diminished.

The 96-well microplates based crystal violet staining (Stepanovic et al., 2000) was used to quantify the biofilm formation by the wild type and the mutant strains in 10% TSB (Figure 3B). As seen with the staining of the pellicle ring, wild type *E. coli* O104:H4 was categorized as a “strong” biofilm former, with $OD_{570} = 0.86$. The deletion of the *aggA* gene resulted in strong reduction of biomass accumulation. OD_{570} of the $\Delta aggA$ mutant was reduced to 0.29, which resulted in the mutant being categorized as “weak” biofilm former.

Effect of O104:H4 AAF/I fimbriae on adherence to produce and abiotic surface. To examine the effect of the deletion of the *aggA* gene on the colonization ability of *E. coli* O104:H4 cells on fresh produce, we first examined the ability of the WT and mutant strains to adhere to the surface of baby spinach and romaine lettuce. In these experiments, fresh spinach and romaine lettuce leaves were co-inoculated with equal amounts of GFP labeled WT and mCherry labeled mutant stains. After overnight storage at 4 °C to allow the establishment of stable attachment but prevent cell division and biofilm formation, the leaves of produce were washed with distilled or chlorinated water (followed by DW rinse) and cell counts remaining on the leaves enumerated (Table 2). After overnight storage (No wash), the ratio of mutant and WT cells on both spinach and lettuce reflected that in the initial inoculums, indicating the absence of an effect by the deletion of *aggA* on the initial attachment to produce surface. This ratio, expressed as competitive attachment index (CAI), remained relatively constant ($p>0.05$) after both spinach and lettuce leaves were washed with either distilled or chlorinated water, indicating both WT and the mutant cells were physically detached by water and chemically inactivated by chlorine by the same degree.

The adherence of *E. coli* O104:H4 WT and Δ *aggA* mutant on spinach leaves was also examined using CLSM. After submerging spinach leaves in a cell suspension containing equal amounts of WT and *aggA* deletion mutant, there was no difference in cell densities of the WT and Δ *aggA* mutant attached to the spinach surface (Figure 4A-C). Similarly, approximately same amounts of WT and Δ *aggA* mutant cells were shown attached to glass surface after a contact time of 10 min and rinsing with PBS (Figure 5A-C).

Colonization of WT and *ΔaggA* mutant O104:H4 on spinach and abiotic surfaces.

Fluorescently labeled wild type and *ΔaggA* mutant cells were mixed at 1:1 ratio in 10% TSB medium, and incubated with spinach leaves in Petri dishes or with glass surface in glass bottom Petri dishes at 25 °C for 10 min, 4 h, and 24 h, followed by rinsing in PBS and examination using CLSM. As stated above, the adherence of WT and *ΔaggA* mutant cells on spinach was at the same level, with approximately equal amounts of GFP labeled WT and mCherry labeled mutant attached to the leaves. No sign of cell aggregation was observed at this stage. The same was true for the adherence on glass surface in the glass bottom Petri dishes.

After 4 h incubation at 25 °C, GFP labeled WT cells attached to the glass surface significantly outnumbered that of the mCherry labeled *ΔaggA* mutant cells (Figure 5D-F). This difference was not due to the differential growth between the WT and the mutant as it has been shown that both the WT and the mutant cells grow at the same rate, and that approximately equal cell concentration were seen in the cell suspension. It was evident that the WT cells had started to form multilayered aggregates, while the *ΔaggA* mutant cells were mostly randomly dispersed on the surface. This trend seemed continually maintained. At 24 h of incubation, a continuous multilayered biofilm structure mainly composed of the wild type cells was observed on the glass surface, while the mCherry labeled *ΔaggA* mutant cells were seen sparsely distributed in the biofilm structure (Figure 5G-I). The biofilm of wild type O104:H4 cells was ~7 μm thick.

We observed a similar pattern of wild type and mutant bacterial colonization on spinach leaves as well. After 24 h incubation, just like in case of the experiments with glass surface, the wild type *E. coli* O104:H4 cells showed aggregation, and they outnumbered the mutant *E. coli* O104:H4 Δ AAF/I cells (Figure 4D). The wild type cells were predominantly found in

aggregates, while the majority of the mutant cells were spread out randomly on the epidermis of the spinach leaves.

DISCUSSION

The colonization of intestinal tract followed by aggressive and rapid biofilm formation is the most important step in EAEC pathogenesis (Hicks et al., 1996; Tzipori et al., 1992; Wakimoto et al., 2004). The majority of enteric bacterial pathogens are equipped with target-specific adherence factors that are implicated in the recognition of surface structures on host cells during the early steps of colonization. One of these specific adherence factors are the aggregative adherence fimbriae AAF/I of *E. coli* O104:H4 (Ahmed et al., 2012; Brzuszkiewicz et al., 2011), which is responsible for specific adhesion to extracellular matrix of vertebrate epithelial cells through fibronectin molecules. Although most fimbrial adhesins specifically target receptor molecules present on animal tissues, some have been shown to play important roles in bacterial attaching to and colonizing on plant tissues and on abiotic surfaces. For example, curli fimbriae seemed to significantly influence biofilm formation by *E. coli* on fresh produce and on abiotic surfaces (Patel et al., 2011). Similarly, *E. coli* common pilus (ECP) seems to target the arabinosyl residues in plant cell walls to mediate adhesion to fresh produce (Rossez et al., 2014).

In this study, we examined the role of *E. coli* O104:H4 AAF/I fimbriae in the attachment and ensuing colonization of plant or abiotic surfaces. The deletion of the AAF/I coding gene did not hinder the ability of O104:H4 cells to attach to spinach or glass surfaces, but it greatly reduced the ability of this strain to form multilayered aggregates. Therefore, the AAF/I fimbriae

do not seem to confer the bacterial cells with higher affinity to produce or abiotic surfaces, but they play an important role in the process of surface colonization and biofilm formation.

Although AAF/I fimbriae are critical for biofilm formation of *E. coli* O104:H4 *in vivo* and *in vitro*, there are EAEC strains associated with persistent infection and diarrhea that lack the AAF fimbriae (Pereira et al., 2007). It was also demonstrated that a putative F-like pili might be equally important in the adhesion process during biofilm formation (Pereira et al., 2010). Reisner and colleagues (Reisner et al., 2006) previously showed that the presence of AAF fimbriae, curli or F-like conjugative pili – well known factors that strongly promote biofilm formation – is not a guarantee that given *E. coli* strains will form robust biofilms. Instead, the authors suggested that the biofilm formation was strongly dependent on the environmental circumstances.

The emergence of Shiga toxin-producing variant of the EAEC, enterohemorrhagic enteroaggregative *E. coli* (EHAEC) like *E. coli* O104:H4, is a significant threat to public health (Beutin and Martin, 2012; Bielaszewska et al., 2011). Such novel combinations of potent pathogenicity systems through lateral gene transfer could pose even greater challenges to the future safety of our food supplies. For *E. coli* O104:H4, we demonstrated here that the AAF/I mediated aggregation confers it greater potential to colonize food and food processing environments. The strong biofilm formation by *E. coli* O104:H4 will also greatly increase its persistence in the environments. A better understanding of the process of these emerging pathogens colonizing the food processing environments will greatly contribute to the safety of future food supplies.

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439

Figure legends

Figure 1. Mutant *ΔaggA* construction and confirmation.

A. Schematic presentation of the mutant construction. Arrows indicate the relative position for the annealing of indicated oligonucleotide primers. CTR-5 and CTR3 were used for PCR confirmation shown in (B). Drawing is not in proportion. **B.** PCR confirmation of *aggA* deletion. Lane 1: TrackIt 100 bp DNA ladder; lane2: negative control, using TW16133 genomic DNA as template; lane 3: PCR using genomic DNA from TW16133 *ΔaggA* mutant. The presence of the 500 bp PCR products indicates the successful replacement of *aggA* sequence with *kan* gene.

Figure 2. Growth kinetics of TW16133 and TW16133 *ΔaggA* mutant strains.

A. Growth profiles of TW16133 (green solid line) and the mutant TW16133 *ΔaggA* strain (red dashed line) in LB. The calculated optical doubling time for both strains was 66 minutes. **B.** Competitive growth of TW16133 and TW16133 *ΔaggA* in LB. The CGI was calculated as the ratio of mutant and WT cells in the co-culture, with this ratio being normalized as 1.00 at time of inoculation.

Figure 3. Biofilm formation by TW16133 and TW16133 *ΔaggA* .

A. Crystal violet staining of aero-liquid interface pellicle rings. **B.** Quantification of biomasses in the biofilms of WT and mutant strains using crystal violet staining based assay. The categorization of biofilm formation was based on the measurement the OD₅₇₀ values of crystal-violet-stained biomass as described by Stepanovic et al. (2000).

Figure 4. Attachment and aggregation of GFP-labeled (green) TW16133 and mCherry-labeled (red) TW16133 *ΔaggA* strains on spinach leaves.

Top: Attachment of labeled bacterial cells after 10 min incubation. **A.** GFP-labeled wild type TW16133 cells. **B.** mCherry-labeled TW16133 *ΔaggA* **C.** Merged image. Scale bar: 10 μm.

Bottom: 3-D rendering of GFP-labeled wild type O104:H4 cells in multilayer aggregates after 24 h incubation. Note that the faint red background and the contour of stomata are due to autofluorescence of the leaves. mCherry labeled mutant cells are visible as intense red dots.

Figure 5. Attachment and colonization of GFP-labeled (green) TW16133 and mCherry-labeled (red) TW16133 *ΔaggA* strains on glass surface.

Differentially labeled cells were examined using CLSM after 10 min (top), 4 h (middle), and 24 h (bottom) incubation. Left (A, D, G) GFP-labeled wild type cells; Center (B, E, H) mCherry-labeled mutant cells; Right (C, F, I) Merged image of dual-fluorescent channels. For each image, the main panel represents the top view of the biofilms and the side panel the side view (Z plane). Scale bar: 10 μm.

Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strains Plasmids Primers	Description	Source
TW16133	<i>E. coli</i> O104:H4 2011 outbreak isolate	EHECSC*
FS4179	TW16133 <i>ΔaggA</i> mutant. Kan ^R	This study
pKD4	Kan cassette containing plasmid, Amp ^R , Kan ^R	Datsenko 2000
pKD78	λ Red recombinase expression plasmid, Cm ^R	CGSC*
pGFP-Gen	Gentamicin resistant version of pGFP, Gen ^R	Nagy 2015
pmCherry-Gen	Gentamicin resistant version of pmCherry, Gen ^R	Nagy 2015
AAF-5	Mutagenesis primer CCCAGAGTGACTGGCAGGATGCCAATCAATG ATCGGAAGCATGCTATATTGTGTAGGCTGGA GCTGCTTC	This study
AAF-3	Mutagenesis primer TTTCCGCAAAAGTAACGACAAGTGATCAATG TATTAAAGCCGGTGCAAAGATGGGAATTAGC CATGGTCC	This study
CTR-5	Primer for mutation confirmation TCATGGGATATATCAGCAGG	This study
CTR-3	Primer for mutation confirmation CCTTGTCCAGATAGCCC	This study

*EHECSC: Michigan State University EHEC Stock Center, East Lansing, MI

CGSC: Yale University *E. coli* Genetic Stock Center (CGSC), New Haven, CT.

Table 2. Adherence of *E. coli* O104:H4 WT and $\Delta aggA$ mutant on spinach and lettuce surface

	Cell Counts (CFU/g)		CI*
	WT	<i>ΔaggA</i>	
Spinach			
Inoculum	4.0 ± 0.7x10 ⁵	4.0 ± 0.3x10 ⁵	0.99 ± 0.08
No Wash	1.7 ± 0.3x10 ⁵	1.4 ± 0.3x10 ⁵	0.81 ± 0.19
DW Wash	1.8 ± 0.1x10 ⁴	1.5 ± 0.1x10 ⁴	0.84 ± 0.08
CI Wash	4.9 ± 0.5x10 ³	4.2 ± 0.4x10 ³	0.85 ± 0.10
Lettuce			
Inoculum	4.3 ± 0.4x10 ⁵	4.7 ± 0.5x10 ⁵	1.09 ± 0.16
No Wash	4.2 ± 0.3x10 ⁴	4.1 ± 0.3x10 ⁴	0.98 ± 0.08
DW Wash	5.0 ± 0.1x10 ³	4.4 ± 0.1x10 ³	0.88 ± 0.02
CI Wash	3.6 ± 0.9x10 ³	3.0 ± 0.8x10 ³	0.85 ± 0.22

*Competitive Index (CI) was calculated as the ratio of the mutant and WT cell counts on treated produce. Mean values are not statistically different ($p > 0.05$).

Figure 1.

A

B

Figure 2.

Figure 3.

A

B

Figure 4.

Figure 5.