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Transcriptomic Analysis for Genetic Mechanisms of the Factors Related to Biofilm Formation in *Escherichia coli* O157:H7

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Abstract Two lineages of enterohemorrhagic *Escherichia coli* O157:H7 (EDL933, $Stx1^+$ and $Stx2^+$) and 86-24 ($Stx2^+$) were investigated to determine the genetic basis of biofilm formation on abiotic surfaces. Strain EDL933 formed a robust biofilm while strain 86-24 formed almost no biofilm on either polystyrene plates or polyethylene tubes. Whole-transcriptome profiles of EDL933 versus 86-24 revealed that in the strong biofilm-forming strain, genes involved in curli biosynthesis and cellulose production were significantly induced, whereas genes involved in indole signaling were most repressed. Additionally, 49 phage genes were highly induced and repressed between the two strains. Curli assays using Congo red plates and scanning electron microscopy corroborated the microarray data as the EDL933 strain produced a large amount of curli, while strain 86-24 formed much less curli. Moreover, EDL933 produced 19-fold more cellulose than 86-24, and indole production in EDL933 was two times lower than that of the strain 86-24. Therefore, it appears *E. coli* O157:H7 EDL933 produces more biofilm because of its increased curli and cellulose production and reduced indole production.

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Introduction

Enterohemorrhagic *Escherichia coli* serotype O157:H7 is the most common human pathogen responsible for outbreaks of hemorrhagic colitis causing bloody diarrhea that can lead to the life-threatening hemolytic-uremic syndrome that affects mostly children [4]. Understanding *E. coli* O157:H7 infections is important given that there are approximately 73,000 infections annually in the U.S., directly leading to 2,000 hospitalizations, 60 deaths, and an economic cost up to $405 million (in 2003 dollars) [10].

Depending on the specific strain, *E. coli* serotype O157:H7 can produce both Shiga toxin 1 (Stx1) and 2 (Stx2) that are responsible for hemorrhagic colitis [26]. *E. coli* serotype O157:H7 strain EDL933 was implicated in two outbreaks of hemorrhagic colitis in USA during 1982 [44] and produces both Stx1 and Stx2 toxins [38]. *E. coli* O157:H7 strain 86-24 caused a hemorrhagic colitis outbreak in USA during 1986 [11] and produces only Stx2 [14].

Bacterial biofilms are ubiquitous in natural, medical, and engineering environments [28]. Biofilms have been associated with many chronic infections such as prostatitis, biliary tract infections, and urinary catheter cystitis by pathogenic *E. coli* due to their high resistance to antimicrobial agents [8]. Food-borne microorganisms, such as *E. coli* O157:H7, can readily attach to and form biofilms on various surfaces, such as stainless steel, glass, and polystyrene [34, 35]. The genetic mechanism of biofilm formation of *E. coli* O157:H7 is a complex process and is now beginning to be unveiled. The production of curli fimbriae [22, 35, 36, 40] is a common contributor to the biofilm formation in *E. coli* O157:H7. Diverse proteins also play an important role in the biofilm formation of *E. coli* O157:H7 [21, 31, 45]. Additionally, intercellular signal molecules,

such as autoinducer-2 [2, 46] and indole [18, 19], are involved in biofilm formation of *E. coli* O157:H7.

In this study, we initially observed a significant difference in the biofilm formation of the two enterohemorrhagic *E. coli* O157:H7 strains, EDL933 and 86-24. DNA microarrays were utilized to identify the genetic basis for this difference in biofilm formation. Global gene expression from the microarray data was corroborated by phenotypic assays including those for curli, cellulose, and indole. It was found that EHEC biofilm formation depends mainly on enhanced curli and cellulose production along with reduced indole production.

Materials and Methods

Bacterial Strains, Materials, and Growth Rate Measurements

Two pathogenic strains of enterohemorrhagic *E. coli* O157:H7, strain EDL933 (ATCC43895) [38] and strain 86-24 (kindly provided by Dr. Arul Jayaraman of Texas A&M University) [11] were used. EDL933 was sequenced [27], whereas the strain 86-24 has not been sequenced. Luria–Bertani medium (LB) [37] was used for growth. All chemicals (Congo red, Coomasie brilliant blue, indole, calcofluor, crystal violet, sodium phosphate, and β-mercapto ethanol) were purchased from Sigma-Aldrich Co. (Missouri, USA). Glutaraldehyde, formaldehyde, acetonitrile, amyl alcohol, ethyl alcohol, hydrochloric acid, OsO4, and ρ-dimethylamino-benzaldehyde were purchased from Junsei Chemical Co. (Tokyo, Japan) or Duksan Pure Chemical Co. (Ansan, Korea). All experiments were performed with LB medium at 37°C (human body temperature). The strains were initially streaked from −80°C glycerol stocks and a fresh single colony was inoculated into 25-ml of LB medium in 250-ml flasks and cultured at 250 rpm. Overnight cultures were diluted 1:100 using LB medium. For cell growth measurements, the turbidity was measured at 600 nm (OD_{600}) with a spectrophotometer (UV-160, Shimadzu, Japan). When the value of OD_{600} was above 0.7, the culture sample was diluted into the linear range of 0.2 to 0.7. Each experiment was performed with at least two independent cultures.

Crystal-Violet Biofilm Assay

A static biofilm formation assay was performed in 96-well polystyrene plates (SPL life sciences, Korea) or 14-ml polyethylene test tubes (SPL life sciences) as previously reported [29]. Briefly, cells were inoculated at an initial turbidity at 600 nm of 0.05 and incubated for 24 h without shaking. Cell growth and total biofilm were measured using crystal violet staining. Each data point was averaged from at least 12 replicate wells (six wells from each of at least two independent cultures).

Total RNA Isolation

For the microarray experiments, planktonic cells of both EDL933 and 86-24 were cultured to a turbidity of 4.0 at 600 nm. Due to the very low biofilm formation of 86-24, planktonic cells were used to study the whole transcriptome profiles. Cells were immediately chilled with dry ice and 95% ethanol (to prevent RNA degradation) for 30 s before centrifugation at 13,000×*g* for 2 min; cell pellets were frozen immediately with dry ice and stored at −80°C. RNA was isolated using a Qiagen RNeasy mini Kit described previously [33].

DNA Microarray Analysis

The *E. coli* GeneChip Genome 2.0 Array (Affymetrix, P/N 900551, Santa Clara, USA) was used to study the differential gene expression profile for EDL933 versus 86-24 as described in the Gene Expression Technical Manual. Ten micrograms of total RNA from each sample was converted to cDNA using random primers. The purified cDNA was fragmented using 0.6 U/μg of DNAse I and end-labeled by terminal transferase reaction incorporating a biotinylated dideoxynucleotide. Hybridization was performed for 16 h at 45°C and 60 rpm as described in the GeneChip Expression Analysis Technical Manual (Affymetrix). After hybridization, the chips were stained and washed in a GeneChip Fluidics Station 450 (Affymetrix), scanned using a GeneChip Array scanner 3000 7G (Affymetrix), and the fluorescent intensity of total probe-sets was scaled automatically in the software to an average value of 500. The probe array images were inspected for any image artifact. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of the polyadenosine RNA controls of *Bacillus subtilis* (*lys*, *phe*, *thr*, and *dap*) at different concentrations were used to monitor the labeling and scanning process. A gene was considered differentially expressed when the *P* value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and when the expression ratio was higher (3-fold) than the standard deviations for whole microarray (2.6-fold) as previously used [33]. Gene functions were obtained from the Affymetrix–NetAffx Analysis Center (https://www.affymetrix.com/analysis/netaffx/index.affx). Raw DNA microarray data are available using GEO series accession number GSE19953.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

To corroborate the DNA microarray data and curli assay and to confirm the induction of indole production, qRT-PCR was used to investigate the transcription level of *csgA* (encoding a curlin major subunit) and *tnaA* (encoding indole-synthesizing tryptophanase) using two RNA samples that were independent from DNA microarray samples. Two primers for *csgA* (forward primer 5′-AGATGT TGGTCAGGGCTCAG-3′ and reverse primer 5′-CGTT GTTACCAAAGCCAACC-3′) and two primers for *tnaA* (forward primer 5′-TACACCATTCCGACTCACCA-3′ and reverse primer 5′-CCGTATCGAAGGCTTCTTTG-3′) were used. The expression level of the housekeeping gene *rrsG* (16S rRNA, forward primer 5′-TATTGCACAATG GGCGCAAG-3′ and reverse primer 5′-ACTTAACAAA CCGCCTGCGT-3′) was used to normalize the expression data of interesting genes. The method of qRT-PCR was adapted [20]. qRT-PCR was performed with SYBR Green master mix (Applied Biosystems, Foster City, USA) and ABI 7500 Real-Time PCR System (Applied Biosystems).

Curli Assay

To measure curli production, Congo red plates (LB agar medium containing 20 μg/ml Congo red and 10 μg/ml Coomassie brilliant blue, and 15 g/L agar) were used as described previously to visualize curli production after 24 h incubation [32]. In order to corroborate the plate assay, scanning electron microscopy (SEM) was used based on our previous protocol [22].

Cellulose Assay

A quantitative cellulose assay using calcofluor [24] was used. Cells (2-ml) at a turbidity of 4.0 were centrifuged and re-suspended in 1-ml of 1% tryptone with 16 μg/ml calcofluor and incubated for 2 h at 37°C and at 250 rpm. Bacterial bound calcofluor was removed by centrifugation for 5 min at 17 000×*g*, and the amount of calcofluor unbound to cellulose was determined by measuring the absorbance of the supernatant at 350 nm.

Indole Assay

To measure the concentration of extracellular indole, EDL933 and 86-24 were grown to a turbidity of 4.0 at 600 nm. The turbidity of 4.0 represents the time point of the stationary phase at which the DNA microarray samples were taken. Supernatants of cell culture were directly used after filtration with 0.45 μm filter. Indole concentrations were measured with reverse-phase HPLC using a 100 × 4.6 mm Chromolith Performance RP-18e column (Merck KGaA, Darmstadt, Germany) and elution with H_2O-0.1% (v/v) trifluoroacetic acid and acetonitrile as the mobile phases at a flow rate of 0.5 ml/min (50:50) [15]. Under these conditions, the retention time and the absorbance maximum was 5.1 min/271 nm for indole.

Results

The main goal of this research was to investigate the genetic mechanism of biofilm formation of the two *E. coli* O157:H7 strains, EDL933 (good biofilm former) and 86-24 (poor biofilm former). Due to the difficulty in obtaining biofilm cells of 86-24, DNA microarrays using planktonic cells were initially utilized to predict the phenotypic changes, and phenotypic assays for curli formation, cellulose, and indole production were performed to corroborate the whole transcriptome data.

Biofilm Formation

Biofilm formation of the two *E. coli* O157:H7 strains was tested using 96-well polystyrene plates and polyethylene test tubes. While EDL933 formed robust biofilms, 86-24 formed almost no biofilm on both polystyrene plates (Fig. 1a) and polyethylene tubes (Fig. 1b). The generation times of the two *E. coli* O157:H7 strains were almost identical (0.74 ± 0.02 h for EDL933 and 0.71 ± 0.03 h for 86-24); therefore, the changes in biofilm formation were not due to differences in growth.

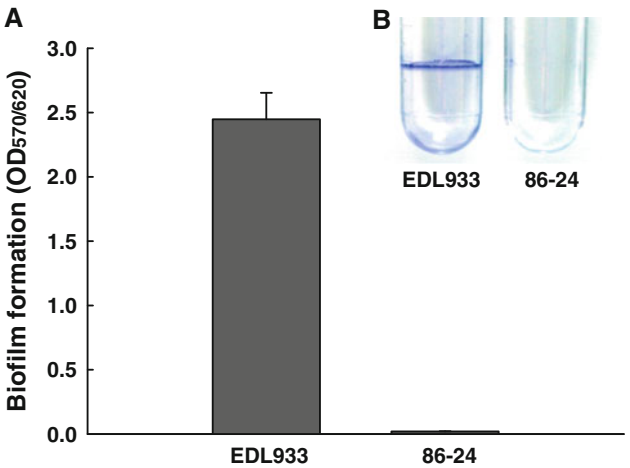

Fig. 1 Biofilm formation of the two *E. coli* O157:H7 strains, EDL933 and 86-24, in 96-well polystyrene plates (**a**) and in polyethylene tubes (**b**) in LB medium without shaking at 37°C after 24 h. At least four independent experiments were conducted (a total 12 wells), and *error bars* indicate one standard deviation

Differential Gene Expression of EDL933 Versus 86-24

To investigate the global genetic basis for the difference in biofilm formation, DNA microarrays were used with planktonic cells of both EDL933 and 86-24. Stationary phase grown planktonic cells ($OD_{600} = 4$) could behave similar to biofilm cells since biofilm gene expression is more closely related to stationary phase planktonic growth [17]. For EDL933 versus 86-24, 436 genes were differentially expressed more than 3-fold; 321 genes were induced and 115 genes were repressed (Supplemental Tables 1 and 2). Partial lists of genes most induced and repressed are shown in the Tables 1 and 2, and the whole set of expression data for the two strains has been deposited in the NCBI GENE Expression Omnibus and are accessible through accession number GSE 19953. As expected, 86-24 showed no signal for the *stx1B* and *stx1A* genes (Table 1) because strain 86-24 lacks Stx1 [14]. Among the differentially expressed genes, many prophage genes of CP-933M, BP-933W, CP-933O, and CP-933V were highly induced in EDL933 (Table 1), whereas prophage genes of CP-933T were highly repressed in EDL933 (Table 2).

The most noticeably induced genes in EDL933 were seven curli genes (*csgBAC* and *csgDEFG*) that were induced 17- to 84-fold (Table 1). The most repressed genes in EDL933 were *trpLED* that were involved in tryptophan (a substrate required for indole synthesis) synthesis and *mtr* that was involved in indole- and tryptophan-uptake (Table 2).

Curli Formation

Curli formation is important for biofilm formation for *E. coli* O157:H7 [31, 35, 36, 40]. Since the microarray data showed that seven curli genes (*csgBAC* and *csgDEFG*) were highly induced (Table 1), curli production was measured using Congo red plates and SEM. Congo red plates were used to observe the curli production with colonies grown under static conditions, while SEM images were used to examine the curli production from planktonic cells that were used for the DNA microarrays and biofilm cells.

EDL933 produced a large amount of curli, whereas 86-24 produced almost no curli on the Congo red plate (Fig. 2a) and with SEM for planktonic cells (Fig. 2b) and biofilm cells (Fig. 2c). For SEM, polymerized curli appeared as 4- to 7-nm wide fibers of varying lengths, and a large amount of curli appeared as a tangled and amorphous matrix surrounding bacterial cells [6]. EDL933 obviously produced a large amount of curli, while 86-24 produced few curli (Fig. 2b, c). Notably, curli formation in EDL933 was more distinctive in biofilm cells than planktonic cells, while there were almost no biofilm cells of 86-24 on a nylon membrane (Fig. 2c). These results corroborate well the microarray data (Table 1). Therefore, these results indicate that curli production is an important indicator of biofilm formations of the two *E. coli* O157:H7 strains. In agreement with this result, a study using transposon mutagenesis also showed that the deletion of curli genes (*csgA*, csgB, or *csgG*) resulted in no biofilm development of *E. coli* O157:H7 [31]. Also, the deletion mutant of *csgA* from the EDL933 strain indirectly reduced biofilm formation [41].

Cellulose Production

The curli protein CsgD also stimulates cellulose production directly by activating transcription of *adrA* [47] and also curli and cellulose play a role in biofilm formation [3, 36, 42]. Since *adrA* was induced 8-fold in EDL933 (Table 1), cellulose production was measured for the two strains using cellulose specific calcofluor [24]. Indeed, EDL933 produced 19-fold more cellulose than 86-24 (0.57 ± 0.08 μg cellulose bound calcofluor/ml cells for EDL933 and 0.03 ± 0.06 μg cellulose bound calcofluor/ml cells for 86-24). Therefore, EDL933 produces much more curli and cellulose than 86-24.

Indole Production

We discovered that extracellular indole inhibits the biofilm formation of *E. coli* O157:H7 by reducing motility, chemotaxis, and attachment to epithelial cells [1, 19]. Since the whole-transcriptome analysis showed that indole-related genes (*trpLED* and *mtr*) were significantly repressed in EDL933 (Table 2), extracellular indole concentrations were measured for the two strains. Corroborating the microarray data, EDL933 produced 2-fold less extracellular indole than 86-24 (210 ± 10 μM for EDL933 vs. 430 ± 10 μM for 86-24). This result partially explains the difference in biofilm formation for these two strains. To investigate any linkage between curli formation and indole production, indole (1 mM) was added to the EDL933 strain but indole did not significantly influence curli formation (data not shown). Also, the previous DNA microarray study with indole addition in EDL933 did not show any change of curli gene expression [1]. Therefore, it appears that mechanisms of indole and curli production are independent.

Confirmation of the Microarray Data

qRT-PCR was used to investigate the transcription of *csgA* (encoding a curlin major subunit) and *tnaA* (encoding indole-synthesizing tryptophanase). The qRT-PCR results were comparable with the DNA microarray data for *csgA* (10.2 ± 0.1-fold induction via qRT-PCR vs. 49-fold in the microarrays). Additionally, qRT-PCR showed 2.6 ± 0.4-

Table 1 Genes *induced* more than 6-fold for planktonic cells of EDL933 versus 86-24

Gene	Fold change	Descriptions
Shiga-like toxin genes		
stx1B[a]	5405	Shiga-like toxin 1 subunit B
stx1A[a]	955	Shiga-like toxin 1 subunit A
Curli and cellulose genes		
csgA	49	Curlin major subunit, coiled surface structures
csgB	79	Minor curlin subunit precursor, similar to CsgA
csgC	84	Putative curli production protein
csgD	17	Putative 2-component transcriptional regulator for curli operon
csgE	24	Curli production assembly transport component,
csgF	28	Curli production assembly transport component
csgG	24	Curli production assembly transport component
adrA	8	Regulated by CsgD involved in cellulose biosynthesis
Prophage genes and unknown genes		
z1215	6	Unknown protein
z1218	8	Hypothetical protein
z1353	6	Putative antirepressor protein of cryptic prophage CP-933M
z1354	9	Putative endopeptidase of cryptic prophage CP-933M
z1362	6	Unknown protein encoded by cryptic prophage CP-933M
z1364	6	Unknown protein encoded by cryptic prophage CP-933M
z1373[a]	23	Unknown protein encoded by cryptic prophage CP-933M
z1443[a]	362	Unknown protein encoded by bacteriophage BP-933W
z1444[a]	1911	Putative serine threonine kinase encoded by bacteriophage BP-933W
z1445[a]	10809	Unknown protein encoded by bacteriophage BP-933W
z1446[a]	1261	Unknown protein encoded by bacteriophage BP-933W
z1448[a]	256	Regulatory protein Cro of bacteriophage BP-933W
z1469[a]	158	Putative lysozyme protein R of bacteriophage BP-933W
z1663	6	Unknown protein
z1811[a]	137	Putative tail component encoded by prophage CP-933N
z1921	6	Unknown protein encoded by prophage CP-933X
z1957[a]	832	Transposase for IS629
z2112[a]	37	Putative ClpP-like protease encoded within prophage CP-933O
z2113	32	Unknown protein encoded within prophage CP-933O
z2114[a]	891	Unknown protein encoded within prophage CP-933O
z2115[a]	16	Unknown protein encoded within prophage CP-933O
z2116[a]	832	Unknown protein encoded within prophage CP-933O
z2117[a]	30	Unknown protein encoded within prophage CP-933O
z3083[a]	69	Putative tail fiber component M of prophage CP-933U
z3341[a]	147	Unknown protein encoded within prophage CP-933V
z3342[a]	69	Unknown protein encoded within prophage CP-933V
z3347[a]	147	Unknown protein encoded within prophage CP-933V
z3348[a]	169	Unknown protein encoded within prophage CP-933V
z3357[a]	549	Putative regulatory protein CII of prophage CP-933V
z3388[a]	208	Unknown protein
z5148	6	Unknown protein
z5878[a]	69	Putative integrase
z5881[a]	338	Unknown protein
z5882[a]	42	Unknown protein
z5883	7	Unknown protein
z5884[a]	91	Unknown protein

Table 1 continued

Gene	Fold change	Descriptions
z5885[a]	13	Putative resolvase
z5886[a]	2195	Unknown protein
z5887[a]	16	Unknown protein
z5888[a]	119	Unknown protein
z5889[a]	119	Unknown protein
ybcK	6	Unknown protein
ybgS	8	Unknown protein
yccT	13	Unknown protein
yhiM	9	Unknown protein
yibH	8	Unknown protein
yohC	6	Unknown protein
Other genes		
aidB	6	Putative acyl coenzyme A
appB	12	Probable third cytochrome oxidase, subunit I
appC	12	Probable third cytochrome oxidase, subunit I
prpB	23	Putative phosphonomutase 2
prpC	17	Putative citrate synthase; propionate metabolism
prpD	21	Hypothetical protein
prpE	18	Putative propionyl-CoA synthetase
rpoS	11	RNA polymerase, sigma S (sigma38) factor
SsuA	28	aliphatic sulfonate binding protein precursor
tfaR	9	Rac prophage

Cells were grown in LB medium at 37°C to a turbidity of 4.0

[a] Gene signals are present in EDL933, but absent in 86-24, otherwise genes are present in both strains. Raw DNA microarray data are available using GEO series accession number GSE19953

fold lower of *tnaA* expression with EDL933 than 86-24, which corroborated the reduced indole production in the EDL933.

Discussion

DNA microarrays have been utilized extensively to obtain whole-transcriptome profiling. For example, two separate lineages of *E. coli* O157:H7 were compared (EDL933 strains LI and LII) to determine if there are differences in expression of virulence-related genes [9]. They found differential expression of *stx2b*, *ureD*, curli (*csgAFEG*), stress-related genes (*hslJ*, *cspG*, *ibpB*, *ibpA*), type III secretion apparatus, LPS, and flagella under anaerobic conditions [9]. Here, we also used DNA microarrays to understand the genetic mechanisms of the biofilm formation of the two *E. coli* O157:H7 strains, EDL933 (ATCC43895) and 86-24. Due to the very low biofilm formation of 86-24, we used planktonic cells of both strains to understand the difference of gene expression between EDL933 and 86-24 and found some meaningful observations. Additional transcriptomic analysis of biofilm cells of both EDL933 and 86-24 would provide more complete insights into *E. coli* O157:H7 biofilm formation.

In this study, about 12% (436 genes) of the total genome (5416 genes, [27]) was differentially expressed (more than 3-fold) probably because of strain-to-strain variations. Among the highly induced and repressed genes (more than 6-fold), 49 prophage genes were identified (Tables 1, 2), which indicates the two tested strains have evolved differently. The antibiotic norfloxacin induces many prophage genes in the BP-933W prophage genome [13] including *z1443* through *z1469* (Table 1), and prophage genes play a role in *E. coli* K-12 biofilm formation [43]. Also, the deletion of the *stx2* or the entire S*tx2*-encoding phage genes in the strain 86-24 did not affect *E. coli* O157:H7 colonization in sheep [7]. Therefore, it would be interesting to investigate whether the EDL933-specific prophage genes (Table 1) affect the biofilm formation and the antibiotic resistance of *E. coli* O157:H7.

The whole transcriptomic profiling showed that curli genes (*csgBAC* and *csgDEFG*), a cellulose gene (*adrA*), and indole-related genes (*trpLED* and *mtr*) are the most differentially expressed loci between the two strains (Table 1). The curli fibers produced by *E. coli* have many of the same biochemical and biophysical properties in common with human amyloid that is associated with Alzheimer's and prion diseases [6], and curli formation is important for biofilm formation of pathogenic *E. coli* O157:H7 [35, 40] as well as non-pathogenic *E. coli* [30]. Using a Congo red assay and SEM, this study confirmed that curli formation is an important positive factor for the biofilm formation of *E. coli* O157:H7 (Fig. 2a, b).

Table 2 Genes *repressed* more than 6-fold for planktonic cells of EDL933 versus 86-24

Gene	Fold change	Descriptions
Prophage genes and unknown genes		
intT[a]	−91	IntT, integrase for prophage CP-933T
z2967[a]	−128	Unknown protein encoded by prophage CP-933T
z2968[a]	−5405	Unknown protein encoded by prophage CP-933T
z2969[a]	−1261	Unknown protein encoded by prophage CP-933T
z2970[a]	−169	Putative regulator for prophage CP-933T
z2971[a]	−8	Unknown protein encoded by prophage CP-933T
z2972[a]	−8	Unknown protein encoded by prophage CP-933T
z2973[a]	−64	Unknown protein encoded by prophage CP-933T
z2974[a]	−23	Unknown protein encoded by prophage CP-933T
z2976[a]	−79	Unknown protein encoded by prophage CP-933T
z2977[a]	−7	Unknown protein encoded by prophage CP-933T
z2979[a]	−12	Putative stability partitioning protein encoded within prophage CP-933T
z2980[a]	−17	Putative stability partitioning protein encoded within CP-933T
z2983[a]	−15	Putative tail fiber assembly protein of prophage CP-933T
z2984[a]	−74	Putative serine acetlyltransferase of prophage CP-933T
z2986[a]	−37	Putative tail fiber protein of prophage CP-933T
z2987[a]	−34	Putative tail fiber component of prophage CP-933T
z2988	−9	Putative tail fiber protein component of prophage CP-933T
z2989[a]	−11	Unknown protein encoded by prophage CP-933T
z2990[a]	−39	Putative tail fiber component of prophage CP-933T
z2991[a]	−39	Putative tail sheath protein of prophage CP-933T
z2992[a]	−34	Putative tail assembly protein of prophage CP-933T
z2994[a]	−315	Unknown protein encoded by prophage CP-933T
z3271	−8	Unknown protein
z5102	−6	Unknown protein
z5111	−6	Unknown protein
ydfV[a]	−23	Unknown protein, Qin prophage
Indole-related genes		
trpD	−12	Anthranilate synthase component II
trpE	−15	Anthranilate synthase component I
trpL	−11	*Trp* operon leader peptide
mtr	−13	Tryptophan-specific transport protein
Other genes		
dxs	−9	Deoxy-xylulose-P synthase
nlpA[a]	−28	Lipoprotein-28

Cells were grown in LB medium at 37°C to a turbidity of 4.0

[a] Gene signals are absent in EDL933, but present in 86-24, otherwise genes are present in both strains. Raw DNA microarray data are available using GEO series accession number GSE19953

Corroborating the high expression of the cellulose gene *adrA* (Table 1), EDL933 produces 19-fold more cellulose than 86-24 although the expression of bacterial cellulose biosynthesis cluster (*bcsABZC*) was not changed in this transcriptome study. Cellulose production increases the binding of *E. coli* O157:H7 to sprouts [25] and increases biofilm formation in *E. coli* K-12 on hydrophilic surfaces, such as glass [24]. Also, cellulose production as well as curli production positively influences the biofilm formation of *E. coli* O157:H7 [36].

In addition, intercellular signal molecules, such as autoinducer-2 [46] and indole [1, 19], are also involved in biofilm formation of *E. coli* O157:H7. Since 86-24 produced more indole than EDL933 and formed almost no biofilm (Fig. 1), this result supports that elevated indole concentrations are partially responsible for the lack of biofilm formation by 86-24. Further support of this is that addition of 1 mM indole to EDL933 reduces its biofilm formation dramatically [19].

Although strain EDL933 and strain 86-24 show different capacities to produce biofilm formation, both the strains caused outbreaks. This study could not identify any positive relationship between virulence gene expression and biofilm formation for the two *E. coli* O157:H7 strains.

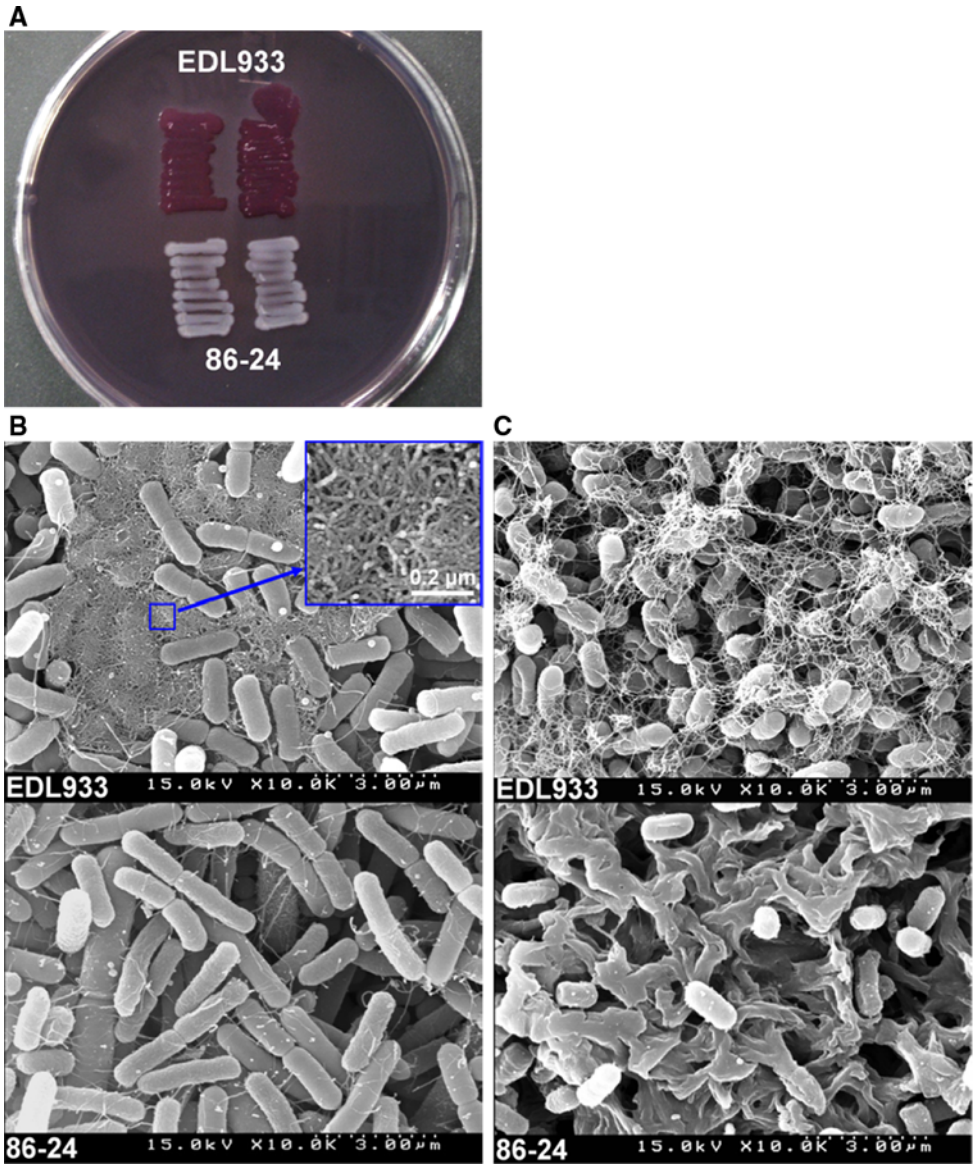

Fig. 2 Curli formation of the two *E. coli* O157:H7 strains, EDL933 and 86-24. Curli production at 37°C after 24 h was observed using Congo red plates (**a**), SEM for planktonic cells (**b**), and biofilm cells grown in a nylon filter (**c**). To show the results of two independent colonies, two streak-outs each for EDL933 and 86-24 were used (**a**). For SEM analysis, planktonic cells were grown in LB medium to turbidity of 2.0 and were directly fixed through the addition of glutaraldehyde and formaldehyde and filtered with a 0.45 μm Nylon filter. Biofilm cells were cultured on a nylon filter in a 96-well plate at 37°C for 24 h. The *inset* shows the magnified amorphous curli/cellulose matrix

Hence, it is possible that environmental cues are missing that induce virulence genes under laboratory conditions within a single species.

To date, no effective therapy for *E. coli* O157:H7 serotypes has been found [4, 39] because antibiotics, anti-motility agents, narcotics, and non-steroidal anti-inflammatory drugs are not usually provided as they increase the risk of developing hemolytic-uremic syndrome, a major cause of acute renal failure in children [39]; these agents induce an SOS response in *E. coli* O157:H7 which induces the prophage-based Shiga toxins [16]. Hence, controlling biofilm formation of *E. coli* O157:H7 is important in medicine as well as in food industry. In addition to anti-microbial therapy, anti-virulence therapies [5, 23] have been proposed because unlike antimicrobials, anti-virulence compounds do not affect growth and so there is less chance of developing resistance [12, 23].

Recently, plant auxin 3-indolylacetonitrile was found to inhibit the biofilm formation of *E. coli* O157:H7 by reducing curli formation and inducing indole production without affecting its growth [22]. The results are consistent with the mechanism of biofilm formation for *E. coli* O157:H7 found here. Since the two tested *E. coli* O157:H7 strains showed same growth rates but showed distinctive biofilm formation due to mainly curli and cellulose formation, controlling either curli formation or cellulose formation are possible ways to prevent biofilm formation of *E. coli* O157:H7.

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