INFECTION AND IMMUNITY, June 2010, p. 2377–2384 Vol. 78, No. 6
0019-9567/10/$12.00 doi:10.1128/IAI.00156-10

Genome-Wide Transposon Mutagenesis Reveals a Role for pO157 Genes in Biofilm Development in *Escherichia coli* O157:H7 EDL933[▽]

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Received 13 February 2010/Returned for modification 2 March 2010/Accepted 19 March 2010

Enterohemorrhagic *Escherichia coli* O157:H7, a world-wide human food-borne pathogen, causes mild to severe diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. The ability of this pathogen to persist in the environment contributes to its dissemination to a wide range of foods and food processing surfaces. Biofilms are thought to be involved in persistence, but the process of biofilm formation is complex and poorly understood in *E. coli* O157:H7. To better understand the genetics of this process, a mini-Tn*5* transposon insertion library was constructed in strain EDL933 and screened for biofilm-negative mutants using a microtiter plate assay. Ninety-five of 11,000 independent insertions (0.86%) were biofilm negative, and transposon insertions were located in 51 distinct genes/intergenic regions that must be involved either directly or indirectly in biofilm formation. All of the 51 biofilm-negative mutants showed reduced biofilm formation on both hydrophilic and hydrophobic surfaces. Thirty-six genes were unique to this study, including genes on the virulence plasmid pO157. The type V secreted autotransporter serine protease EspP and the enterohemolysin translocator EhxD were found to be directly involved in biofilm formation. In addition, EhxD and EspP were also important for adherence to T84 intestinal epithelial cells, suggesting a role for these genes in tissue interactions *in vivo*.

Enterohemorrhagic *Escherichia coli* O157:H7 was first recognized as the probable cause of hemorrhagic colitis in humans in 1982 (50). Since then, this organism has emerged as a major cause of food-borne illness in countries around the world, including the United States (49), Europe (17, 18, 24, 36), Japan (39), and Australia (28). Outbreaks have been associated with a variety of food sources, including ground beef (50), green leafy vegetables (1, 41), and nonpasteurized milk (26), and environments such as municipal water and lakes (27, 55). Symptoms in infected humans range from mild diarrhea to severe, hemorrhagic colitis, with 5 to 10% of patients developing hemolytic uremic syndrome (HUS), making *E. coli* O157:H7 one of the leading causes of acute renal failure in children and the elderly (38).

Early studies have shown that some strains of *E. coli* O157:H7 form biofilms on both abiotic and biotic surfaces outside the host (15, 51, 58). Biofilms are exopolymeric matrix-enclosed bacterial populations that are firmly adherent to each other and/or to surfaces (9). Biofilms have been shown to be responsible for protection from a variety of environmental stresses, such as acidification, high temperatures, and desiccation (52). Moreover, microbes in biofilms are highly resistant to other adverse conditions, such as sanitizers and household cleaners (44, 45) as well as antibiotics (23, 34). The ability of this pathogen to form biofilms on a wide range of food surfaces as well as food processing surfaces makes *E. coli* O157:H7 problematic in both the health and food industries (29).

Biofilm formation is a dynamic and complex process and includes initial attachment of cells to the substratum, physiological changes within the organism, multiplication of the cells to form microcolonies, and eventually maturation of the biofilm (42). Because of this complexity, the process of biofilm formation and its regulation is poorly understood. Previous studies in *E. coli* O157:H7 have focused on individual genes and the specific genetic pathways that are responsible for biofilm formation (10, 51, 58, 60). In contrast, few studies have focused on studying genetic factors that control *E. coli* O157:H7 biofilm formation on a global scale, although studies of this type have been performed with other bacterial pathogens (43, 47, 59).

The goal of this study was to gain additional insights into biofilm formation in *E. coli* O157:H7. A global mutational approach with a mini-Tn*5* transposon was used to study the process of biofilm formation in strain EDL933, a strong biofilm-forming strain. This strain was first isolated during a multistate outbreak involving contaminated hamburgers (50). A library of >11,000 mutants was generated and screened for a biofilm-negative phenotype. Our results reinforced the fact that biofilm formation is a complex process involving a large number of genes and genetic pathways. This study discovered several pO157 genes that were not previously known to be linked to biofilm formation.

MATERIALS AND METHODS

Bacteria. The *E. coli* strains used in this study are shown in Table 1. A spontaneous nalidixic acid-resistant mutant of *Escherichia coli* O157:H7 strain EDL933 was selected to serve as a counterselection for these studies. The mutation in this mutant had no effect on biofilm formation or on the ability of EDL933 to colonize and persist in a sheep model of colonization (unpublished data). For all biofilm assays, the cultures were grown in Luria-Bertani (LB) broth for 24 h at 30°C under stationary conditions. For all other experiments, the cultures were grown overnight in LB broth at 37°C with shaking at 200 to 220 rpm. The temperature-sensitive *E. coli* strain harboring the pRedET plasmid

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▽ Published ahead of print on 29 March 2010.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
BW19795	RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant/*srlC300 creC510 hsdR17 endA1 zbf-5 uidA*(ΔMluI)::*pir*$^+$ *thi*	G. Phillips
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
ISM1536	*E. coli* O157:H7 EDL933	N. Cornick
ISM1191	*E. coli* O157:H7 EDL933, Nal^r	This study
ISM1205	BW19795 pUTminiTn*5*Km2; Amp^r Kan^r	40
ISM1230	*E. coli* O157:H7 EDL933 lacking plasmid pO157	N. Cornick
ISM1893	ISM1191/miniTn*5*Km2 in *hlyB*, Nal^r Kan^r	This study
ISM2014	ISM1536 Δ*hlyA* Kan^r	This study
ISM1211	ISM1536 Δ*hlyB* Kan^r	This study
ISM1216	ISM1536 Δ*hlyD* Kan^r	This study
ISM2013	ISM1216/pISM30 Amp^r Kan^r	This study
ISM1978	ISM1536/pISM31 Spc^r	This study
ISM2015	ISM1230/pISM31 Spc^r	This study
ISM1944	ISM1893/pISM31 Spc^r Kan^r	This study
ISM2016	ISM 2014/pISM31 Spc^r Kan^r	This study
ISM2017	ISM1211/pISM31 Spc^r Kan^r	This study
ISM2018	ISM1216/pISM31 Spc^r Kan^r	This study
ISM2019	ISM2013/pISM31 Spc^r Kan^r	This study
ISM1881	ISM1191/miniTn*5*Km2 in L7020, Nal^r Kan^r	This study
ISM1919	ISM1191/miniTn*5*Km2 in *hlyC hlyA*, Nal^r Kan^r	This study
ISM1227	ISM1536 ΔL7020 Kan^r	This study
Plasmids		
pKD4	Derivative of pANTSγ that contains an FLP recombination target-flanked Kan^r gene from pCP15	13
pRedET	Derivative of pSC101, Amp^r, temp sensitive, carries lambda red recombinase	Gene Bridges
pBAD18	Expression vector, Amp^r	Beckwith lab
pISM30	pBAD18 expressing *ehxCABD* operon	This study
pISM31	pMHE6 expressing GFPuv	G. Phillips

[a] Nal^r, nalidixic acid resistant; Amp^r, ampicillin resistant; Kan^r, kanamycin resistant; Spc^r, spectinomycin resistant.

(Gene Bridges GmbH, Dresden, Germany) was grown at 30°C. Antibiotic concentrations were 100 μg/ml for ampicillin, 50 μg/ml for kanamycin, and 20 μg/ml for nalidixic acid except where noted. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO). The growth of individual mutants was assessed by measuring the growth endpoint optical density at 600 nm (OD_{600}) after 24 h of growth in 96-well plates and compared to that of wild-type EDL933.

Mutant Library Construction. Transposon mutagenesis was performed as described previously (22) with a few modifications. To obtain random mini-Tn*5*Km2 insertion mutants, the conjugal donor strain *E. coli* BW19795 containing the plasmid pUTmini-Tn*5*Km2 (supplied by David Holden, Imperial College, London, United Kingdom) was conjugated with *E. coli* O157:H7 EDL933. One milliliter of overnight culture of each strain was pelleted, washed three times with phosphate-buffered saline (PBS), resuspended in 5 ml of antibiotic-free LB broth, and incubated at 37°C with shaking until an OD_{600} of 0.7 to 1.0 was reached. The BW19795 donor strain was then transferred to stationary conditions for 30 min to allow regeneration of pili. Two hundred microliters of each strain was combined, and the mating mixture was plated onto a sterile membrane placed on a stack of sterile filter paper. Once the liquid medium was removed by capillary action, the membrane was transferred to an LB agar plate cell side up. Following overnight incubation at 37°C, membranes were vortexed with LB medium, and the suspension was incubated with shaking for 1 h at 37°C and then plated in 100-μl aliquots on LB plates containing kanamycin plus nalidixic acid as a counterselection against the donor strain. Each plate yielded 300 to 400 colonies of kanamycin-resistant mutants. Colonies were picked into 96-well plates, and each was scored for ampicillin resistance. The ampicillin-sensitive mutants were rearrayed into 96-well plates and stored at −70°C for further analysis. A total of 11,000 independent mutants were obtained.

Assays for biofilm formation. The screen for the biofilm-negative phenotype was performed using a microtiter plate assay as described previously (16) with minor modifications. This assay is based on the ability of biofilm-forming bacteria to adhere to the wells of a 96-well microtiter plate, which are subsequently visualized by staining with crystal violet. Each plate of mini-Tn*5* insertion mutants from an overnight LB culture was replica plated using a 96-prong replicator into fresh Costar 96-well, flat-bottom, nontreated polystyrene plates (Corning, Inc., New York, NY) containing 150 μl of LB broth per well. After 24 h of incubation at 30°C under stationary conditions, the plate was rinsed twice with water, and the adherent bacteria were stained with 0.01% crystal violet (175 μl/well) for 20 min. After staining, the plates were washed again twice with water. At this point, biofilms were visible as a violet ring on the side of each well and as a generalized staining of the well (Fig. 1). Mutants that lacked ring formation were scored as having a biofilm-negative phenotype. For growth assessment, a duplicate plate incubated overnight was briefly vigorously shaken on a microtiter plate shaker, and the OD_{600} was measured and compared with that of the wild-type control strain. Strains with growth that differed from wild-type growth ($P < 0.01$) were not considered further, as were mutants that showed inconsistent biofilm formation. In each plate, *E. coli* strain BW19795 was used as a negative control, and a well containing medium only was used as a blank.

For the quantitative biofilm assay (57), 12- by 75-mm polystyrene tubes (Fisher) were used. An overnight culture of each mutant, plus controls, was diluted 1:100 into 2 ml of LB broth and incubated at 30°C under stationary conditions for 24 h. The tubes were then rinsed twice with water and stained with 2.5 ml of 0.01% crystal violet for 20 min. After being washed three times with

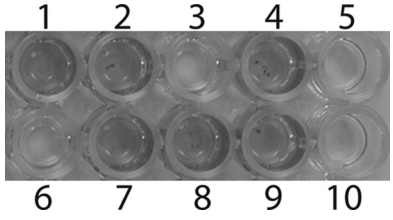

FIG. 1. Microtiter plate assay showing screening of mini-Tn*5*Km2 mutants for the biofilm-negative phenotype. Wells 4 and 9 contained the wild-type biofilm-positive cells, wells 5 and 10 contained the biofilm-negative control (BW19795), wells 3 and 6 contained biofilm-negative transposon mutants, and wells 1, 2, 7, and 8 contained biofilm-positive transposon mutants.

TABLE 2. Primers used in this study

Primer	Sequence (5′ → 3′)
P6 U	CGAGCTCGAATTCGGCCTAG
P7 U	CTGCAGGCATGCAAGCTTCG
P6 M	GCCAGATCTGATCAAGAGAC
P7 M	GCCGAACTTGTGTATAAGAGTC
Tn5	GGCCAGATCTGATCAAGAGA
Y-Linker	CTGCTCGAATTCAAGCTTCT
Test F	TGTGTAGGCTGGAGCTGCTTC
ehxA F	AGTAAAAAACAGACAAGATTTT AATTTTAATATTGAGAAAGAA AACTAATTGTGTAGGCTGGA GCTGCTTC
ehxA R	TATAACGATGACCATTCCTCCTG GAATGGCCATCACCTCCTCTT TTAGTCCATATGAATATCCTC CTTA
ehxA test R	AGAACTACATTTACTCATCA
ehxB F	AGACTAAAAGAGGAGGTGATG GCCATTCCAGGAGGAATGGT CATCGTTATTGTGTAGGCTGG AGCTGCTTC
ehxB R	TTATAACGGCAAACCAAATCCC ATAAACCTTTCATGTAAAAGC GCATACGCATATGAATATCCT CCTTA
ehxB test R	AGACATCAGAAAAAACCGTC
ehxD F	CCAAAAAAGACAGTTTATATGC ATATTTATATCAGTTGCAGGC ATAACGTTGTGTAGGCTGGA GCTGCTTC
ehxD R	ATTATTTCAGAAATCTATATCAT ATAAAAAGCCAATATGTTATT TATATAATATGAATATCCTC CTTA
ehxD test R	ACATAGATAA TCTTTACAAG
L7020F	ATGAATAAAA TATACTCTCT TAAATACAGC CATATTACAG GAGGGTTAATCGCTGTTTCTT GTGTAGGCTGGAGCTGCTTC
L7020 R	GCAATTTTAT TGAAAGACTG ATTATATCCG TTCAGGTCAA GAGTTCCGCCATTTTTCGCAC ATATGAATATCCTCCTTA
L7020 test R	TACAGCACCTGAATCAGTTG CA
ehx kpn1 F	CTCAGGTACCTAGATGCTTCTT GCTTAAAA
ehx xba1 R	GATCTCTAGACTAACGTTCACG TAAACTTT

water, tubes were air dried and destained with 2.5 ml of 80% ethyl alcohol for 15 min. The tubes were vortexed, 100 μl was transferred to a new 96-well plate, and the optical density was measured at 595 nm using a Spectra MAX 190 spectrophotometer (Molecular Devices, Union City, CA). The optical density measurements were used as a measure of relative amounts of biofilms formed. All experiments were performed in triplicate.

Identification of mini-Tn*5* insertion sites. The mini-Tn*5* insertion sites in biofilm-negative mutants were mapped by sequencing the amplified genomic region at the site of mini-Tn*5* insertion. The amplification of the region of the DNA at the site of transposon insertion was done using either single-primer PCR(25) or Y-Linker PCR(30). The PCR products were then sequenced and analyzed using BLAST against the *E. coli* O157: H7 EDL933 genome sequence.

Construction of deletion mutants. To generate deletion mutations, a one-step gene inactivation method adapted from that described by Datsenko and Wanner (13) was used. The temperature-sensitive plasmid pRedET (Gene Bridges, Dresden, Germany), encoding lambda red recombinase, was transformed into *E. coli* O157:H7 EDL933. The kanamycin resistance gene was amplified from pKD4 (13) using primers shown in Table 2. Each primer sequence contained target-homologous sequences as well as sequences for amplification of the kanamycin gene. The products of this reaction were electroporated (2,000 V, 129 Ω) using a BTX electrocell manipulator (model 600; Harvard Apparatus, Holliston, MA) into *E. coli* O157:H7 EDL933/pRedET that was previously induced with 0.4% L-arabinose for 1 h. The cells were incubated in SOC medium (20 g tryptone, 5 g yeast extract, 2 g $MgCl_2 \cdot 6H_2O$, 2.5 g $MgSO_4 \cdot 7H_2O$, and 3.6 g glucose per liter; pH 7.5) for 1 h and then plated on selective medium (LB supplemented with 25 μg/ml of kanamycin) at 37°C.

Confirmation of mutant constructions and determination of the locations of the kanamycin gene insertions were done by PCR. Primer F (which has homology within the kanamycin cassette) and primer R (which has homology immediately downstream of the gene sequences that were being replaced) were used to generate PCR products. To ensure curation of the temperature-sensitive pRedET plasmid, confirmed mutants were first grown at 42°C for 2 h and then plated on LB plates and incubated overnight at 37°C. The isolated colonies were picked and screened for kanamycin resistance and ampicillin sensitivity. All of the primers used are shown in Table 2.

Construction of plasmid pISM30 for genetic complementation. The *ehxCABD* operon was amplified using primers that also incorporated upstream KpnI and downstream XbaI restriction sites (Table 2). The PCR was done using LongAmpTaq DNA polymerase (New England Biolabs) with the following parameters: 2 min at 95°C; 30 cycles of 95°C for 30 s, 58°C for 7 min, and 72°C for 1 min; and 5 min of extension at 72°C. The fragments were digested with KpnI and XbaI, cloned into vector pBAD18, and then transformed into *E. coli* DH5α. One plasmid, designated pISM30, was confirmed by restriction digestion and was electroporated into *E. coli* O157:H7 EDL933 Δ*ehxD*.

Cell culture. The T84 human colonic adenocarcinoma cells were maintained in 25-cm^2 (Falcon) tissue culture flasks as monolayers at 37°C with 5% CO_2. The cell cultures were grown in Dulbecco modified Eagle medium (DMEM)–F-12 medium (Invitrogen) supplemented with 2.5 mM L-glutamine, 5% fetal bovine serum, and gentamicin (50 μg/ml). The cells were passed every 7 days by treatment with 0.5% trypsin, and the medium was replaced every other day.

Bacterial adhesion assay. Quantitative adhesion assays were performed using monolayers of T84 cells grown on glass coverslips. The glass coverslips were treated with 1 N HCl for 10 min, washed three times with sterile water, and placed in six-well polystyrene tissue culture plates (Costar, Corning, Corning, NY). T84 cells (4×10^5) were seeded onto the glass coverslips in each well and allowed to attach overnight. The monolayers were then washed with Hanks balanced salt solution and replenished with 1 ml of culture medium without antibiotics. An overnight culture of bacteria was diluted 1:20 in fresh LB and grown for another 2 h. One hundred microliters of this culture (approximately 4×10^6 bacteria) was added to each well containing T84 monolayer cultures. Bacterial cultures were serially diluted and plated to enumerate bacteria added. The tissue culture plates were then incubated at 37°C with 5% CO_2 for 1.5 h. The coverslips were washed three times with PBS to remove nonadhered bacteria, and then the glass coverslip was transferred to a fresh six-well tissue culture plate. The T84 cells were then detached and lysed using 1 ml of 0.1% Triton X-100 for 15 min. In preliminary studies, this concentration of Triton X-100 had no effect on viability of *E. coli* O157:H7 EDL933. This solution was serially diluted in PBS and spread onto LB agar to enumerate the bacteria adhered to T84 cells. The percentage of adherence was calculated as number of bacteria adhered/number of bacteria added to the well × 100, and the relative percentage of adherence was calculated as percentage of adherence of mutant/percentage of adherence of wild type × 100. All experiments were done in triplicate. The paired Student *t* test was performed to identify statistical differences.

For microscopic analysis, bacteria were transformed with pISM31, a derivative of pMHE6 (20) expressing GFPuv (12). The T84 cells were seeded as described above and grown for 48 h until they were semiconfluent. Bacterial cultures were prepared, and the adherence assays were performed as described above. The plates were then incubated at 37°C with 5% CO_2 for 1.5 h. Following this, the coverslips were washed three times with PBS, and the cells were fixed using 4% paraformaldehyde in PBS for 10 min. The coverslips were washed twice with PBS, and treated with BSP buffer (250 mg bovine serum albumin and 100 mg saponin per 100 ml PBS) for 5 min, and then washed twice with BSP. The cells were stained for F-actin (54) with Alexa Fluor 546-labeled phalloidin (Invitrogen) (1:200 dilution in BSP) for 1 h, washed twice with BSP, and then mounted on a glass slide using mounting solution with DAPI (4′,6′-diamidino-2-phenylindole). Once the slides were dry, the coverslips were sealed using clear nail polish, and images were captured using green and red filters on an Olympus IX70 inverted fluorescence microscope equipped with a DP70 digital camera. The images were merged using ImageJ software (National Institutes of Health).

RESULTS

Identification of biofilm genes in *E. coli* O157:H7 EDL933. In previous studies, *E. coli* O157:H7 EDL933 was shown to form biofilms on inert surfaces (15). Since there have been no reports of studies performed on a global scale to identify biofilm-linked genes in *E. coli* O157:H7, we conducted a global mutational study using mini-Tn*5*Km2 to identify genes involved either directly or indirectly in biofilm formation. During the initial screen, 114 mutants (1.04% of the total library) had a biofilm-negative phenotype (Fig. 1). Following the confirmation of the phenotype, the growth of each strain was assessed. After elimination of mutants with inconsistent biofilm formation or a growth deficiency, 95 mutants that were biofilm negative (0.86% of the library) were studied further. For convenience, these mutants were designated *b*iofilm-*n*egative *p*henotype (Bnp) mutants.

The precise mini-Tn*5* insertion sites of these Bnp mutants were identified by DNA sequencing. Our results indicated that there was only a single transposon insertion within the genome in each mutant. This was evident from the Y-Linker PCR, which showed amplification of only one fragment, and from the DNA sequence trace chromatograms, which gave a single sequence. The mini-Tn*5* insertion sites in 19 of the Bnp mutants could not be identified due to a failure to amplify the region at the transposon insertion sites despite repeated attempts.

The 76 insertions that could be identified were distributed randomly throughout the genome of *E. coli* O157:H7 EDL933. In some cases, there were multiple insertions in the same gene coding region but at different locations. Fifty-one distinct genes/intergenic regions were identified (Table 3). Thirty-two insertions were in coding sequences of known function, and 19 were in hypothetical genes whose functions are yet to be assigned. Twenty-five insertions occurred in sequences having homology to *E. coli* K-12 sequences, and 19 occurred in sequences not shared with K-12 (O islands); three are on the pO157 plasmid, and five are phage carried. The functions of these genes vary; they include genes that encode structural components (*ecpD*, *csgG*, *csgB*, *csgA*, *tolQ*, *waaL*, and *waaP*), enzymes (*yahF*, *yaiH*, *galU*, *cls*, *manC*, *wbdQ*, *fcl*, *aroC*, *relA*, *rfaC*, and *dsbA*), regulators (Z2086, *yihF*, and *hns*), receptors (Z1178, Z0700, and Z3635), and hypothetical proteins. Three independent insertions were identified in the curli pilus operon (*csgG*, *csgB*, and *csgA*) and four independent insertions in the lipopolysaccharide (LPS) biosynthesis operon (*waaL*, *waaP*, *waaD*, and *waaJ*).

Plasmid pO157 genes involved in biofilm formation. In our mutational analysis we identified three independent insertions of mini-Tn*5*km2 in pO157, one in the type V secreted serine protease *espP* (7) and two in the enterohemolysin operon *ehxCABD* (3, 4) (one in *ehxB* and one between *ehxC* and *ehxA*). We confirmed the role of pO157 in biofilm formation by testing strain ISM1230, a derivative of EDL933 lacking the plasmid. This strain failed to make biofilms (Fig. 2). To confirm the linkage between specific genes and the biofilm phenotype, deletion mutants were constructed for three of the genes that were biofilm negative during screening, i.e., *espP*, *ehxB*, and *ehxA*. The deletion mutants were then compared for their growth and biofilm phenotype using a quantitative tube assay with the corresponding mini-Tn*5*km2 Bnp mutants (Bnp6, Bnp18, and Bnp44, respectively) (Fig. 2). Two of the deletion mutants (the Δ*ehxB* and Δ*ehxA* mutants) showed no difference in biofilm formation compared to the wild type, but their corresponding mini-Tn*5*km2 Bnp mutants, Bnp18 and Bnp44, were deficient in biofilm formation. The other deletion-insertion mutant (the Δ*espP* mutant) behaved in manner similar to that of its corresponding mini-Tn*5*km2 Bnp mutant (Bnp6). One explanation for the inconsistency in *ehxCABD* mutations is that the insertion of the transposon caused polarity effects on downstream expression of *ehxD* (8) that would not occur with the deletion mutations. All deletions were constructed in frame, and the kanamycin marker used in the deletion reaction lacks a transcriptional stop signal, allowing for continued transcription and expression of downstream genes. Thus, these results indicate that *ehxD*, and not *ehxA* or *ehxB*, may be the critical element in biofilm formation. To test this hypothesis, we constructed a Δ*ehxD* mutant and tested it for the biofilm phenotype. Our quantitative assay showed that the deletion of *ehxD* caused a negative biofilm phenotype (Fig. 2). To further confirm this linkage, we constructed a plasmid for genetic complementation by cloning the *ehxCABD* operon under control of its native promoter into plasmid pBAD18. When this plasmid was transformed in the Δ*ehxD* mutant (Δ*ehxD*+pISM30), the biofilm phenotype was restored to levels comparable to those for the wild type (Fig. 2).

Role of pO157 genes in adherence to T84 cells. To analyze the role of *ehxCABD* in adherence to T84 cells, wild-type *E. coli* O157:H7 EDL933, the wild type without pO157, and the Bnp6, Bnp18, Bnp44, Δ*espP*, Δ*ehxA*, Δ*ehxB*, Δ*ehxD*, Δ*ehxD*+pISM30 mutants were tested for adherence to T84 cells. Both microscopic and quantitative analyses were done. For microscopic analysis, all of the bacterial strains were transformed with a green fluorescent protein-expressing plasmid, pISM31. As shown in Fig. 3, wild-type *E. coli* O157:H7 EDL933 and the Δ*ehxA*, Δ*ehxB*, and Δ*ehxD*+pISM30 mutants adhered to T84 cells, while *E. coli* O157:H7 EDL933 without pO157 and the Bnp6, Bnp18, Bnp44, Δ*espP*, and Δ*ehxD* mutants failed to adhere. The quantitative analysis also showed that there is no significant difference in relative adherence to T84 cells between wild-type *E. coli* O157:H7 EDL933 and the Δ*ehxA*, Δ*ehxB*, and Δ*ehxD*+pISM30 mutants but that there was a significant reduction in the relative adherence of *E. coli* O157:H7 EDL933 without pO157, and the Bnp6, Bnp18, Bnp44, Δ*espP*, and Δ*ehxD* mutants (Fig. 4). For this experiment, the percentage of wild-type bacteria adhering to the T84 monolayers was 32.6%. Strains with less than 4% adherence were considered negative.

DISCUSSION

Earlier studies with *Pseudomonas fluorescens* and *Staphylococcus aureus* showed that the process of biofilm formation is complex and involves several convergent and divergent pathways (43, 59). To obtain an overall idea of the genes required for biofilm formation in the human pathogen *E. coli* O157:H7 EDL933, a global mutagenesis approach with mini-Tn*5*km2 was performed.

Previous studies with *E. coli* have shown that Tn*5* and its minitransposon derivatives insert randomly in the genome

TABLE 3. Mini-Tn*5* transposon biofilm-negative mutants of *E. coli* O157:H7 EDL933[e]

Mutant	Locus tag	Gene name	Product or function
Bnp1[a]	Z3917[b]		Hypothetical protein
Bnp2[a]	Z4881[b]		Putative aldolase
Bnp3[a]	Z5856		Putative aspartate carbamoyltransferase
Bnp4[a]	Z2256[b]		Unknown protein associated with Rhs element
Bnp5[a]	Z5890[b]		Partial putative integrase
Bnp6[a]	L7020[d]	*espP*	Putative exoprotein precursor
Bnp7[a]	Z3635		Putative receptor protein
Bnp8[a]	Z3182[b]	*hisD*	L-histidinal:NAD^+ oxidoreductase
Bnp9[a]	Z4625	*acrE*	Protein affects cell membrane permeability
Bnp10	Z0472	*yaiH*	Putative enzyme
Bnp11[a]	Z1555[b]		Hypothetical protein
Bnp12[a]	Z2436	*ynaJ*	Hypothetical protein
Bnp13[a]	Z1921[c]		Unknown protein encoded by CP933X
Bnp14[a]	Z0151	*ecpD*	Putative fimbrial chaperone protein
Bnp15[a]	Z1456[c]/Z1457[c]		Hypothetical protein encoded by BP-933W/putative DNA binding protein of BP-933W
Bnp16	Z3592	*aroC*	Chorismate synthase
Bnp17	Z2026	*cls*	Cardiolipin synthase
Bnp18[a]	L7049[d]	*hlyB*	Hemolysin transport protein
Bnp19[a]	Z3497 [b]	*glpQ*	Glycerophosphodiester phosphodiesterase
Bnp20	Z4099	*relA*	(p)ppGpp synthetase I
Bnp21	Z5049	*waaL*	Surface polymer ligase
Bnp22[a]	Z3199[b]/Z3200[b]	*wbdP*/*per*	Putative glycosyl transferase/perosamine synthetase
Bnp23	Z5050[b]	*waaD*	LPS biosynthesis enzyme
Bnp24[a]	Z2163	*ydeH*	Hypothetical protein
Bnp25	Z1675/Z1676	*csgB*/*csgA*	Minor curlin subunit precursor/curlin major subunit, coiled surface structures
Bnp26[a]	Z2086[c]		Putative regulator
Bnp27[a]	Z3660	*yfeA*	Hypothetical protein
Bnp28[a]	Z5214[b]		Hypothetical protein
Bnp29	Z5051[b]	*waaJ*	LPS α1,2-glucosyltransferase
Bnp30	Z1670	*csgG*	Curli production assembly/transport component
Bnp31[a]	Z1212[b]/Z1213[b]		Hypothetical protein
Bnp32[a]	Z1213[b]		Hypothetical protein
Bnp33	Z2012/Z2013	*galU*/*hns*	Glucose-1-phosphate uridylyltransferase/DNA binding protein; pleiotropic regulator
Bnp34	Z3195/Z3196	*manC*/*wbdQ*	Mannose-1-phosphate guanosyltransferase/GDP-mannose mannosylhydrolase
Bnp35[a]	Z0340[c]		Unknown protein encoded in prophage CP933I
Bnp36	Z1676	*csgA*	Curlin major subunit, coiled surface structures
Bnp37	Z0905	*tolQ*	Inner membrane protein
Bnp38	Z5054	*waaP*	LPS biosynthesis enzyme
Bnp39[a]	Z4328[b]		Hypothetical protein
Bnp40[a]	Z0700[b]		Putative receptor
Bnp41[a]	Z3918[b]		Putative chaperone protein
Bnp42[a]	Z1494[c]		Unknown protein encoded by BP-933W
Bnp43[a]	Z0021[b]		Hypothetical protein
Bnp44[a]	L7047/L7048[d]	*hlyC*/*hlyA*	Hemolysin protein
Bnp45[a]	Z1977	*ychM*	Hypothetical protein
Bnp46[a]	Z0408/Z0409	*yahE*/*yahF*	Hypothetical protein/putative oxidoreductase subunit
Bnp47[a]	Z3197	*fcl*	Fucose synthetase
Bnp48[a]	Z0904	*ybgC*	Hypothetical protein
Bnp49[a]	Z4327[b]		Hypothetical protein
Bnp50	Z5392	*dsbA*	Protein disulfide isomerase I
Bnp51[a]	Z1177[b]/Z1178[b]		Partial putative phage inhibition protein/putative receptor

[a] Genes not previously shown to be involved with biofilm formation.
[b] On O islands.
[c] Carried in prophage.
[d] On pO157.
[e] A slash indicates that the transposon site was between the genes indicated.

(14). Based on the assumption that there are 1,000 essential genes out of 5,361 open reading frames in the genome of *E. coli* O157:H7 EDL933 (46), the generation of approximately 11,000 random mutants should give at least a 99% probability of inactivating 90% of nonessential genes. These studies uncovered several new genes that are involved in biofilm formation. Our approach was not complete, however, as we also missed some genes already shown to be involved in biofilm formation in *E. coli* O157:H7, such as *csgD*, *ompA*, and *cadA* (58). This is because our screen was not saturating. In fact, approximately 55,000 random mutants would need to be generated for inactivation of 100% of all nonessential genes with a 99% probability.

This study identified 51 Bnp genes in *E. coli* O157:H7, of which 19 were O-island pathogen-associated genes, five were phage carried, and three were located on pO157 (Table 3). This suggests that some of the regulatory pathways involved in biofilm formation in *E. coli* O157:H7 are unique to that sero-

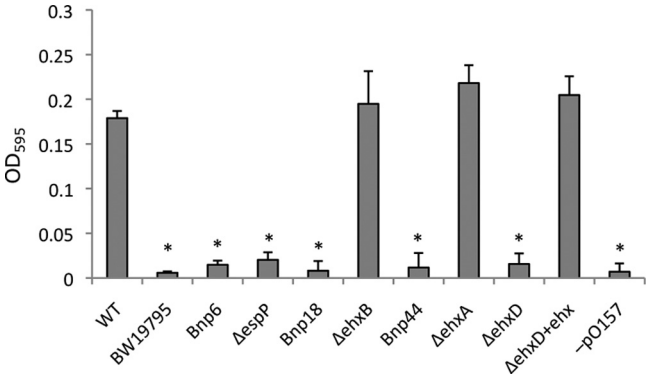

FIG. 2. Comparison of biofilm formation on polystyrene by *E. coli* O157:H7 EDL933 transposon insertion and deletion mutants along with wild-type (WT) and negative-control (BW19795) strains. Data represent means plus standard errors for three replicates. Bnp6, *espP*::Tn*5*; Bnp18, *ehxB*::Tn*5*; Bnp44, Tn*5* inserted at the *ehxC-ehxA* junction; Δ*ehxD*+*ehx*, Δ*ehxD* complemented with pISM30. *, significantly different from the wild-type control ($P < 0.01$).

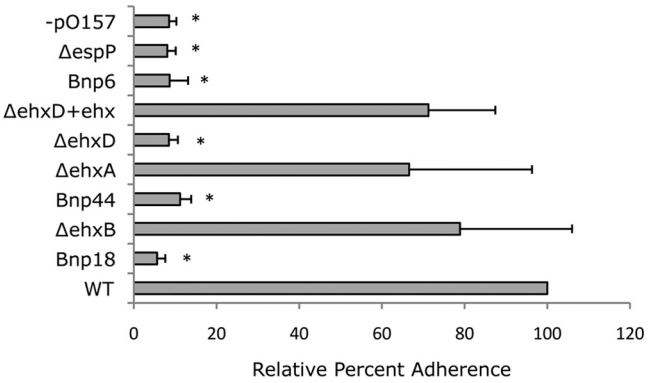

FIG. 4. Adherence of wild-type *E. coli* O157:H7 EDL933 and transposon insertion and deletion mutants to T84 cells shown quantitatively. Data represent means plus standard errors for three replicates. *, significantly different from the wild-type control ($P < 0.01$).

type. Previous studies have shown that Shiga toxin-producing *E. coli* strains, including the O157:H7 serotype, are retained or persist in the ruminant gastrointestinal tract better than other *E. coli* pathotypes (11). The reason for this is unknown, but it is possible that genes unique to the O157:H7 serotype that are involved in biofilm development may enhance persistence in specific environmental situations such as those encountered in the bovine gastrointestinal tract or on food processing surfaces.

Of the 51 Bnp genes identified, 20 have defined functions and 31 either are hypothetical or have only putative functions assigned. Among those 20 genes with known functions are those already demonstrated to have a role in biofilm formation in other organisms. *relA* was shown to be required for efficient biofilm formation in *Listeria monocytogenes*, *Streptococcus mutans*, and *E. coli* (2, 32, 56). It has also been demonstrated that *relA* mutants show lower levels of (p)ppGpp and higher levels of LuxS under amino acid starvation conditions (32, 56). The changes in the levels of LuxS and (p)ppGpp affect pathways that are required for biofilm formation. Our study showed that in *E. coli* O157:H7, *relA* is also involved in biofilm formation. A more thorough transcriptional analysis of this gene may lead to the discovery of additional determinants of biofilm formation for *E. coli* O157:H7.

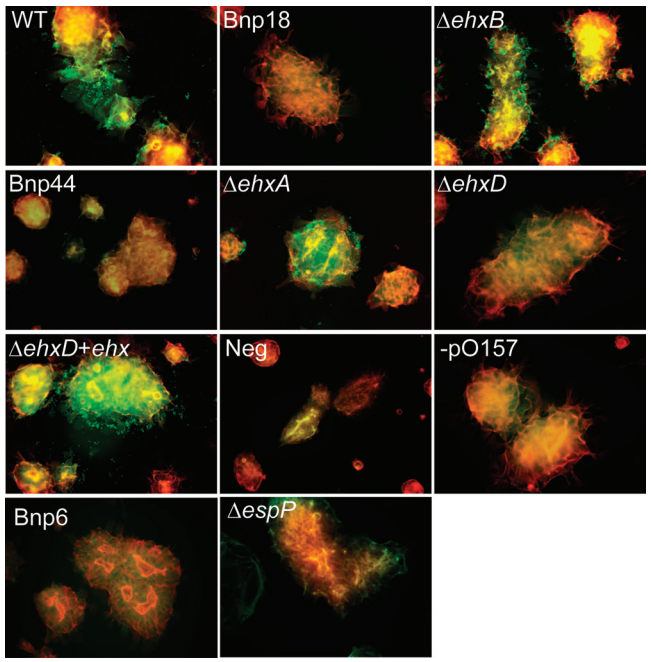

FIG. 3. Fluorescence microscopic pictures of GFPuv, expressing wild-type *E. coli* O157:H7 EDL933 and transposon insertion and deletion mutants, adhering to T84 cells. All pictures are merged images of DAPI (blue), F-actin (orange), and GFPuv-expressing bacterium (green) staining.

Four independent insertions in the lipopolysaccharide (LPS) biosynthesis operon (*waaL*, *waaP*, *waaD*, and *waaJ*) that resulted in the loss of biofilm formation were identified. In Gram-negative bacteria, LPS influences the physiochemical characteristics of the cell surface. In *Pseudomonas aeruginosa* it has been shown that the production of A band or B band LPS influences the surface characteristics and modifies the binding capabilities of the bacterium (35). The presence of four independent insertions in the LPS biosynthesis operon strongly suggests that LPS is directly involved in biofilm formation in *E. coli* O157:H7 as well. Thus, a more thorough study of this operon is needed to more accurately describe its role in biofilm development.

In *Vibrio cholerae*, *galU*, another Bnp gene, was shown to be essential for the formation of biofilms (5). In several organisms, including *E. coli* O157:H7, *galU* encodes glucose-1-phosphate uridylyl transferase and is responsible for synthesis of UDP glucose. The synthesis of UDP galactose via UDP-glucose is necessary for biosynthesis of exopolysaccharide, which is a binding substrate in biofilms. In *Streptococcus mutans*, *aroC* (chorismate synthase) has been shown to be involved in biofilm formation (53). *cls* (cardiolipin synthase) has also been shown to be involved in biofilm formation in *Mycobacterium ulcerans* (37). Lee et al. showed that disulfide bond isomerase A (*dsbA*) was involved in biofilm formation on abiotic surfaces in *E. coli* O157:H7 and that Δ*dsbA* strains were reduced in attachment to HT-29 epithelial cells and virulence in *Caenorhabditis elegans* (31).

Another set of interesting genes identified in this study are the *csg* genes, which are responsible for the production of the curli pilus, a coiled surface structure produced by various microbes. Two operons, *csgBA* and *csgDEFG*, are necessary for curli formation. *csgA* encodes the curlin subunit, CsgB is thought to nucleate CsgA curlin fibers, CsgD is a transcriptional activator of the *csgBA* operon, and CsgE, CsgF, and CsgG are three putative curli assembly factors (61). Curli pili are highly adhesive proteinaceous structures required for bacterial adherence to surfaces as well as bacterium-to-bacterium binding (62), and they are critical for both primary surface colonization and subsequent biofilm development in the formation of microcolonies (48). More-detailed genetic studies showed that CsgD is a control unit for biofilm formation and coordinates both positive (*csgAB* and *yaiC*) and negative (*pepD* and *yagS*) determinants of biofilm formation (6, 21). It is possible that the curli pilin subunit (CsgA and CsgB) is directly involved in the attachment of the organism to the surface and that the regulator CsgD is involved in the maturation of the biofilm. Although this gene is one of the best-studied biofilm-associated genes in *E. coli* O157:H7, questions remain about the actual mechanism of attachment of organisms to the substratum through curli pili.

Several Bnp genes identified in this study (Table 3) are uniquely associated with biofilm formation. Most of these either are hypothetical genes or have been assigned putative functions based on homology studies. Determination of how these genes might function in the development or maintenance of biofilms will require further study.

Three of the 51 Bnp genes obtained in the initial screening, Bnp6, Bnp18, and Bnp44, were chosen for further study because of their location on the plasmid pO157. A role of translocator EhxD in biofilm formation was confirmed by deleting the gene *ehxD* (strain ISM1216) and complementing the biofilm-negative phenotype with plasmid pISM30, expressing the *ehxCABD* operon (strain ISM2013). The role of *ehxD* in biofilm formation is unknown and needs further study. One possible hypothesis is the translocator EhxD functions independently of EhxB and transports factors that are critical to biofilm formation. Our study is the first to show the involvement of the plasmid pO157-carried enterohemolysin operon *ehxCABD* in biofilm formation in *E. coli* O157:H7, although a recent study reported the importance of pO157 in biofilm development (33). In addition to *ehxD*, *espP* was also important to biofilm development. We can only speculate on how this type V secreted serine protease (7, 19) might be involved in biofilm formation, but it must play an important role.

We were also interested in testing whether *ehxD* and *espP* might have a role in adherence to T84 colonic adenocarcinoma cells, testing the hypothesis that biofilm formation and cellular adherence to epithelial cells are linked in *E. coli* O157. A previous study by Dziva et al. showed that EspP was critical to adherence to a bovine primary rectal epithelial cell line (19), so it was reasonable to think that other products of genes on pO157 might also be involved in cell adherence. Clearly, cellular adherence occurs to T84 cells *in vitro* at 1 h after inoculation (Fig. 4). The loss of *ehxD* or *espP*, however, resulted in *E. coli* O157:H7 being incapable of T84 cell adherence. This was also true for a pO157-negative strain. Complementation of the Δ*ehxD* strain resulted in adherence, suggesting that this protein, along with EspP (19), has an important role in cellular adherence and possibly tissue interactions *in vivo*.

In summary, through random mutagenesis we were able to identify genes not previously known to be involved in biofilm formation in *E. coli* O157. Two of these genes, *espP* and *ehxD*, are located on the virulence plasmid pO157. In addition to biofilm formation, these genes are important for adherence to T84 colonic epithelial cells. Further analysis of these gene products and the pathways involved will provide a better understanding of the process of biofilm formation and colonization by *E. coli* O157:H7, which in turn should help in the development of methods to decrease its prevalence in food animals and to control its dissemination in the environment.

ACKNOWLEDGMENTS

This study was funded in part by the U.S. Department of Agriculture, Agricultural Research Service grant no. 58-3625-4-173, and by the Iowa State University Institute for Food Safety and Security.

We thank Gregory Phillips for assistance with deletion mutagenesis and the plasmid pISM31 and Bryan Bellaire for assistance with microscopy.

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Editor: S. M. Payne