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Characterization of Enteropathogenic *Escherichia coli* Mutants That Fail To Disrupt Host Cell Spreading and Attachment to Substratum

Chen Nadler, Yulia Shifrin, Shani Nov, Simi Kobi, and Ilan Rosenshine*

Department of Molecular Genetics and Biotechnology, The Hebrew University, Faculty of Medicine, POB 12272, Jerusalem 91120, Israel

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Upon infection of host cells, enteropathogenic *Escherichia coli* (EPEC) delivers a set of effector proteins into the host cell cytoplasm via the type III secretion system (TTSS). The effectors subvert various host cell functions. We found that EPEC interferes with the spreading and ultimately with the attachment of suspended fibroblasts or epithelial cells, and we isolated mini-Tn*10*kan insertion mutants that failed to similarly affect host cells. In most mutants, the insertion sites were mapped to genes encoding TTSS components, including *cesD*, *escC*, *escJ*, *escV*, *espD*, *sepL*, *espB*, and *escF*. Other mutants contained insertions in *micC* or upstream of *bfpP*, *yehL*, or *ydeP*. The insertion upstream of *ydeP* was associated with a reduction in TTSS protein production and was studied further. To determine whether the apparent repression was due to constitutive expression of the downstream encoded genes, *ydeP* and *ydeO* expression vectors were constructed. Expression of recombinant YdeP, YdeO, or EvgA, a positive regulator of both *ydeP* and *ydeO*, repressed TTSS protein production. Our results suggest that upon activation of the EvgAS two-component system, EvgA (the response regulator) activates both *ydeP* and *ydeO* expression and that YdeP and YdeO act conjointly, directly or indirectly repressing expression of the TTSS genes.

Enteropathogenic *Escherichia coli* (EPEC) causes infantile diarrhea, which results in the death of several hundred thousand children each year in developing countries (10). EPEC infection is characterized by intimate binding to intestinal enterocytes, localized effacement of absorptive microvilli, transient filopodium formation, and accumulation of polymerized actin beneath the bacteria (26, 46, 54). This histopathology is termed the attaching and effacing lesion (18, 28). The genes responsible for attaching and effacing lesion formation are clustered in the locus of enterocyte effacement (LEE) (37). The LEE consists of 41 genes encoding a type III secretion system (TTSS), as well as several effector proteins. The LEE genes are organized in five major operons, *LEE1* to *LEE5* (16, 17). Operons *LEE2* to *LEE5* are positively regulated by Ler, which is encoded by the *LEE1* operon (19, 38). Regulation of the *LEE1* operon is complex and involves many factors, including H-NS (3, 56), integration host factor (19), Fis (21), PerC (3, 38), BipA (23), GadX (48), GrlA, and GrlR (9), as well as quorum sensing (27, 49). In the closely related organism enterohemorrhagic *E. coli* (EHEC), the *LEE* operons are regulated by EtrA, EivF, RpoS, and ClpX (25, 63). For efficient attachment, EPEC uses the plasmid-encoded bundle-forming pilus (BFP) (18, 46). The BFP is encoded by two operons, *perABC* (*bfpTVW*) and *bfpA-L*, which contains 13 genes encoding the pilus structural genes (22, 53).

E. coli K-12 has 32 two-component systems (39), one of which is the EvgAS system, whose physiological role is obscure. EvgS is the sensor histidine kinase, and EvgA is the corresponding response regulator (40, 43, 44). The transcriptome of an *E. coli* K-12 *evgAS* mutant has been compared with that of the wild type, using DNA array analysis (42). In addition, the attempts to identify the *evgAS* mutant phenotype have included a high-throughput phenotype microarray analysis (64), in which nearly 2,000 different phenotypes were examined. However, neither study showed that there was a significant difference between the *evgAS* mutant and the wild-type strain. Additionally, an in vitro examination revealed low levels of EvgS autophosphorylation and EvgA transphosphorylation (59). The results of these studies may imply that the EvgAS two-component system does not function in *E. coli* or that the input signal for *evgAS* activation has not been found.

Here we show that EPEC prevents spreading and ultimately interferes with the attachment of suspended fibroblasts, or epithelial cells, to the substratum. Furthermore, we isolated mini-Tn*10*kan insertion mutants deficient in inhibition of host cell attachment. These mutants contain insertions in LEE genes, including *cesD*, *escC*, *escJ*, *escV*, *espD*, *sepL*, *espB*, and *escF*, in non-LEE genes, including *micC*, and in the regions upstream of *bfpP*, *yehL*, and *ydeP*. Further characterization of the insertion upstream of *ydeP* indicated that it is associated with a reduction in TTSS protein production, probably as a result of constitutive expression of *ydeP* and *ydeO*. Our results suggest that upon EvgA activation, *ydeP* and *ydeO* are expressed and possibly act jointly, directly or indirectly, to repress the expression of the TTSS genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. The strains and plasmids used in this study are listed in Table 1, and the oligonucleotides used are listed in Table 2. Bacteria were grown in either LB broth, M9 minimal medium (35), or Dulbecco's modified Eagle's medium (DMEM) (Sigma). When indicated below, the medium was supplemented with either streptomycin (100 μg/ml), tetracycline (10 μg/ml), ampicillin (50 μg/ml), kanamycin (50 μg/ml), or chloramphenicol (25 μg/ml).

* Corresponding author. Mailing address: Department of Molecular Genetics and Biotechnology, Faculty of Medicine, The Hebrew University, POB 12272, Jerusalem 91120, Israel. Phone: 972 2 6758754. Fax: 972 2 6757308. E-mail: ilanro@cc.huji.ac.il.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype[a]	Reference or source
Strains		
E2348/69	EPEC (strain O127:H6) isolated from an outbreak in Taunton, United Kingdom, St^r	32
27-3-2(1)	EPEC Δ*escN*::*TnphoA* St^r Kan^r Nal^s	11
DF2	E2348/69 Δ*ler*::*kan*	19
SM10λpir	*E. coli thi-1 thi leu tonA lacY supE recA*::RP4-2-Tc::Mu, λpir, Tet^r Kan^r	M. S. Donnenberg
DY378	W3110 λ*cI857* Δ(*cro-bioA*)	62
SA42	*Salmonella enterica* serovar Typhimurium SL1344 *hemA26*::Tn*10d*-tet	15
SN116	E2348/69 with mini-Tn*10*kan insertion upstream of *ydeP*	This study
CNY1794	E2348/69 Δ*evgA*::*kan*	This study
CNY1929	E2348/69 Δ*evgA*::*cm*	This study
CNY2092	E2348/69 Δ*ydeP*::*kan*	This study
CNY2077	E2348/69 Δ*ydeO*::*kan*	This study
CNY2075	E2348/69 Δ*ydeP-b1500-ydeO*::*kan*	This study
CNY2288	E2348/69 Δ*evgA*::*cm* Δ*gadX*::*tet*	This study
CNY2289	E2348/69 Δ*evgA*::*kan* Δ*gadXW*::*tet*	This study
CNY2290	E2348/69 Δ*ydeO*::*kan* Δ*gadX*::*tet*	This study
CNY2291	E2348/69 Δ*ydeO*::*kan* Δ*gadXW*::*tet*	This study
Plasmids		
pLOF/Km	mini-Tn*10*kan delivery plasmid, Kan^r Amp^r	8
pUC18	Cloning vector	60
pKD46	pBAD promoter 30°C sensitive replicon, Amp^r	7
pKD4	Template plasmid, Kan^r Amp^r	7
pKD3	Template plasmid, Cm^r Amp^r	7
pSA10	pKK177-3 derivative containing $lacI^q$	45
pCNY2441	pSA10 carrying *evgA*, Amp^r	This study
pCNY1743	pSA10 carrying *ydeP*, Amp^r	This study
pCNY1696	pSA10 carrying *b1500*, Amp^r	This study
pCNY1697	pSA10 carrying *ydeO*, Amp^r	This study
pCNY1698	pSA10 carrying *yhiF*, Amp^r	This study
pTU14	pACYC184 encoding $lacI^q$ and P*tac-ler*	2

[a] St^r, streptomycin resistance; Kan^r, kanamycin resistance; Nal^s, nalidixic acid sensitivity; Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

Analysis of inhibition of host cell spreading and attachment. Bacterial cultures that were grown overnight at 37°C in LB broth without shaking were diluted 1:50 in DMEM and grown without shaking under conditions known to stimulate TTSS expression for 3.5 h in the presence of 5% CO_2 at 37°C to the mid-logarithmic growth phase (optical density at 600 nm, ~0.3), which created preactivated cultures (19). Prior to infection, DU17 fibroblasts (47) were trypsinized, washed with phosphate-buffered saline (PBS), and resuspended in DMEM supplemented with 10% fetal calf serum. A 500-μl suspension of DU17 cells (~10^5 fibroblasts) was infected with 500 μl of a culture of preactivated bacteria (~5×10^7 bacteria) at a multiplicity of infection (MOI) of ~1:500. The infected cells were seeded onto coverslips in wells of 24-well plates. After 15, 60, or 180 min of infection, cells were fixed for 10 min using 3.7% paraformaldehyde in PBS, washed, permeabilized for 2 min with 0.1% Triton X-100, rewashed, and actin stained using phalloidin-rhodamine (Sigma). Slides were prepared, and micrographs were obtained by fluorescence microscopy.

Mutagenesis and isolation of mutants deficient in blocking host cell attachment. EPEC strain E2348/69 was mutated using a mini-Tn*10*kan transposon that was delivered into EPEC strain E2348/69 (St^r) by mating with *E. coli* SM10λpir (Kan^r) containing pLOF/Km (Amp^r Kan^r), as previously described (8). Transconjugate EPEC colonies were selected on LB medium plates supplemented with kanamycin and streptomycin and tested for ampicillin sensitivity to confirm the lack of plasmid integration into the EPEC chromosome. Each mutated colony was inoculated into LB broth in 96-well tissue plates (master plates) and grown overnight at 37°C. The cultures were then preactivated, maintaining the 96-well format. Concurrently, DU17 fibroblast cultures were trypsinized, washed, and resuspended in DMEM supplemented with 10% fetal calf serum. Using a 96-well plate, 50 μl of suspended DU17 cells (~3.4×10^4 fibroblasts) was inoculated with 50-μl portions of different mutants (~5×10^6 bacteria) (MOI, ~1:66). DU17 was infected with each mutant in duplicate. Infection with wild-type EPEC and infection with the *escN* mutant served as positive and negative controls, respectively. After 1 h of infection at 37°C, the wells were washed three times with PBS, stained with 100 μl Giemsa stain (Sigma) for 10 min, and then washed with PBS to remove the excess Giemsa stain. The wells were then examined. An inability to block cell attachment resulted in blue staining of a well. Mutants that were unable to block host cell attachment were recovered from the master plates.

Quantitative attachment assay. Using 24-well tissue culture plates, 500 μl of suspended DU17 cells (~2.5×10^5 fibroblasts) was infected with 500 μl of preactivated cells of different mutants (~5×10^7 bacteria) (MOI, 1:50). Specific wells in each plate in which the cells were infected with wild-type EPEC and with the *escN* mutant served as positive and negative controls, respectively. After 1 h of infection at 37°C, the wells were washed three times with DMEM and incubated for 20 min with 200 μl lysis buffer (0.1% Triton X-100, 20 mM $MgCl_2$), which specifically lysed the DU17 cells. Next, 180 μl of the lysate was cleared by 5 min of centrifugation ($174 \times g$). The protein concentration of the cleared lysate was directly correlated with the number of attached DU17 cells (data not shown). The protein concentration was measured using a BCA protein assay kit (Sigma). Assays were carried out in triplicate, and standard errors were calculated. The effect of each mutant was compared with the effects of the controls; the effect of wild-type EPEC was defined as 0%, and the effect of the *escN* mutant was defined as 100%.

Determination of the mini-Tn*10*kan insertion sites. All mutants were analyzed by PCR using primers for various LEE genes (6) and for the mini-Tn*10*kan cassette (C1_Tn10_out_R and C4_Tn10_out_F), followed by sequencing (Table 2). DNA fragments containing insertions in non-LEE mutants were cloned. To this end, the total DNA of each mutant was digested with either EcoRI, PstI, MfeI, or SalI and subjected to Southern analysis. As a probe, we used a digoxigenin-labeled kanamycin cassette generated by PCR, using primers C2_Tn10_in_F and C3_Tn10_in_R (Table 2) and a DIG kit (Roche). Digested fragments smaller than 5 kbp were purified from the agarose gel and ligated into pUC18, using either EcoRI, PstI, MfeI, or SalI depending on the enzyme used for genomic DNA fragmentation. Clones containing mini-Tn*10*kan were selected using LB agar plates supplemented with kanamycin. Sequencing using primers M13_F and M13_R revealed the exact location of the mini-Tn*10*kan insertion.

Construction of mutants and plasmids. Specific genes were deleted with the aid of the λ red system, using the DY378 strain or the pKD46 plasmid, as previously described (7, 62). We used pKD3, pKD4 (7), and the chromosomal DNA of *Salmonella* containing Tn*10d*-Tet (15) as chloramphenicol, kanamycin,

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Restriction enzyme
Oligonucleotides used for mini-Tn*10*kan mutant characterization		
C1_Tn10_out_R	CGCGAGCCCATTTATACC	
C2_Tn10_in_F	GTCGGGCAATCAGGTGCG	
C3_Tn10_in_R	GGAGAAAACTCACCGAGGC	
C4_Tn10_out_F	GGTATTGATAATCCTGATATG	
M13_F	CGCCAGGGTTTTCCCAGTCACGAC	
M13_R	AGCGGATAACAATTTCACACAGGA	
pSA10_F	GCTCGTATAATGTGTGGAATTG	
pSA10_R	CGATGGTGTCAACGTAAATGC	
Oligonucleotides used for cloning and sequencing		
*evgA*_F	GCGAATTCATGAACGCAATAATTATTGATG	EcoRI
*evgA*_R	CGCTGCAGTTAGCCGATTTTGTTACGTT	PstI
*ydeP*_F	GCCAATTGATGAAGAAAAAAATTGAATCC	MfeI
*ydeP*_R	CAGTCGAC*TTA***GTGATGGTGATGGTGATG**ATTTGATGGTTCTAATTCAACC	SalI
pre_*ydeP*_F	GCTCCATTCACGAAAATTGC	
*ydeP*_R4	CTTTACCCACACGGATACTCG	
*ydeP*_F2	CGACTGCTCCAACATGTGC	
*ydeP*_R3	GTACATTAGAGTGACCTCGTAGTGG	
*ydeP*_F3	CCAGAACGTACAGCAACTGG	
*ydeP*_R2	GGATGCGCTGGTTATAGTCG	
*ydeP*_F4	CTGTGCTGCCAGAGTTTGC	
*b1500*_F	GCGAATTCATGCATGCGACCATAGTG	EcoRI
*b1500*_R	CACTGCAGTCAGTCTTGCAAACTATTGATAATG	PstI
*ydeO*_F	GCGAATTCATGTCGCTCGTTTGTTCTG	EcoRI
*ydeO*_R	CACTGCAGCTAAAGCATTCATCGTGTTGTC	PstI
Oligonucleotides used for generation and analysis of gene inactivation		
evgA K/O_F	CTGTATTACTACAGGGAGAAGGGAAATGCTTCATTGCAAAGGGAATAATCTgtgtaggctggagctgcttc	
evgA K/O_R	CTTATGGTCGACCAAAGACCACAACAGAGAAGAAAAATATAGGGTAAAAAcatatgaatatcctcctta	
ydeP K/O_F	ATCATCGCTATTACAAATCCTAATAATTCATTTCCACACAGGATAAGCAGgtgtaggctggagctgcttc	
ydeP K/O_R	ATTTGATGGTTCTAATTCAACCGGAATACTTTTATAGCCAGGAATGCCACcatatgaatatcctcctta	
ydeO K/O_F	GAAATGTTAAAAAAGTATCGATAAAAACTTTATTGTTTTAAGGAGATAAAAgtgtaggctggagctgcttc	
ydeO K/O_R	TACGCAGCGTGTGTGGTTGACTAGTCGTTAGCAAATAATCAAATAGCTAAcatatgaatatcctcctta	
gadX K/O_F	CGGCGTGCTACATTAATAAACAGTAATATGTTTATGTAATATTAAGTCAACTgtgtaggctggagctgcttc	
gadX K/O_R	TCCTCTTCCCGGTCCCCTATGCCGGGTTTTTTTTATGTCTGAGTAAAACTcatatgaatatcctcctta	
gadW K/O_F	TAGTATACTGACATTGAAATAATCGCAGTAATGAAATATAAGGAATAGTCgtgtaggctggagctgcttc	
gadW K/O_R	TCACATGAAGCAGACGTGAGATCCTGACCAATATTCAAATGCGAAATATGcatatgaatatcctcctta	
pre *evgA*_F	CAATTCTTACGCCTGTAGGATTAGTG	
post *evgA*_R	CTTCTCTGTTGTGGTCTTTGG	
pre *ydeP*_F	GCTCCATTCACGAAAATTGC	
post *ydeO*_R	CAGCTTTACATCATCTGCCG	
pre *gadX*_F	TCCTGTTTTCCCGCTTCG	
post *gadW*_R	GAACTAGCCGGTAGCTCGTC	
K1_pKD_out	CAGTCATAGCCGAATAGCCT	
K2_pKD_out	CGGTGCCCTGAATGAACTGC	

[a] Restriction enzyme sites are underlined, a stop codon is indicated by italics, a six-His-encoding sequence is indicated by boldface type, and priming site sequences of template plasmids used for gene inactivation are indicated by lowercase type.

and tetracycline DNA template-resistant cassettes, respectively. The primers used for mutant analysis are shown in Table 2. In some cases, mutations were first generated in DY378 and then transferred into wild-type EPEC harboring pKD46. To generate the expression plasmids, genes were amplified with specific primers (Table 2) and cloned into the pSA10 cloning vector (45) as previously described (2). The plasmid sequence was verified by sequencing, using analysis primers pSA10_F and pSA10_R (Table 2).

Analysis of protein production and secretion under conditions stimulating TTSS and BFP production. One milliliter of preactivated bacteria was centrifuged, and supernatants containing the secreted proteins, as well as pellets containing total proteins, were recovered. Where indicated below, isopropyl-β-D-thiogalactopyranoside (IPTG) was added 1 h after dilution in DMEM. The protein levels in the different samples were adjusted according to the cell density, and the samples were used for immunoblot analysis. Membranes were incubated with different primary polyclonal rabbit antibodies (anti-EspB, -Tir, -intimin, -Ler, -EscJ, -BfpA, or -SSB) diluted in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl; pH 7.5) containing 1% bovine serum albumin. Binding of secondary anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma) was detected using BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium (Promega).

Conditions used for comparison of the *evgA* mutant with wild-type EPEC. To create TTSS-repressing conditions, we compared EspB production in overnight

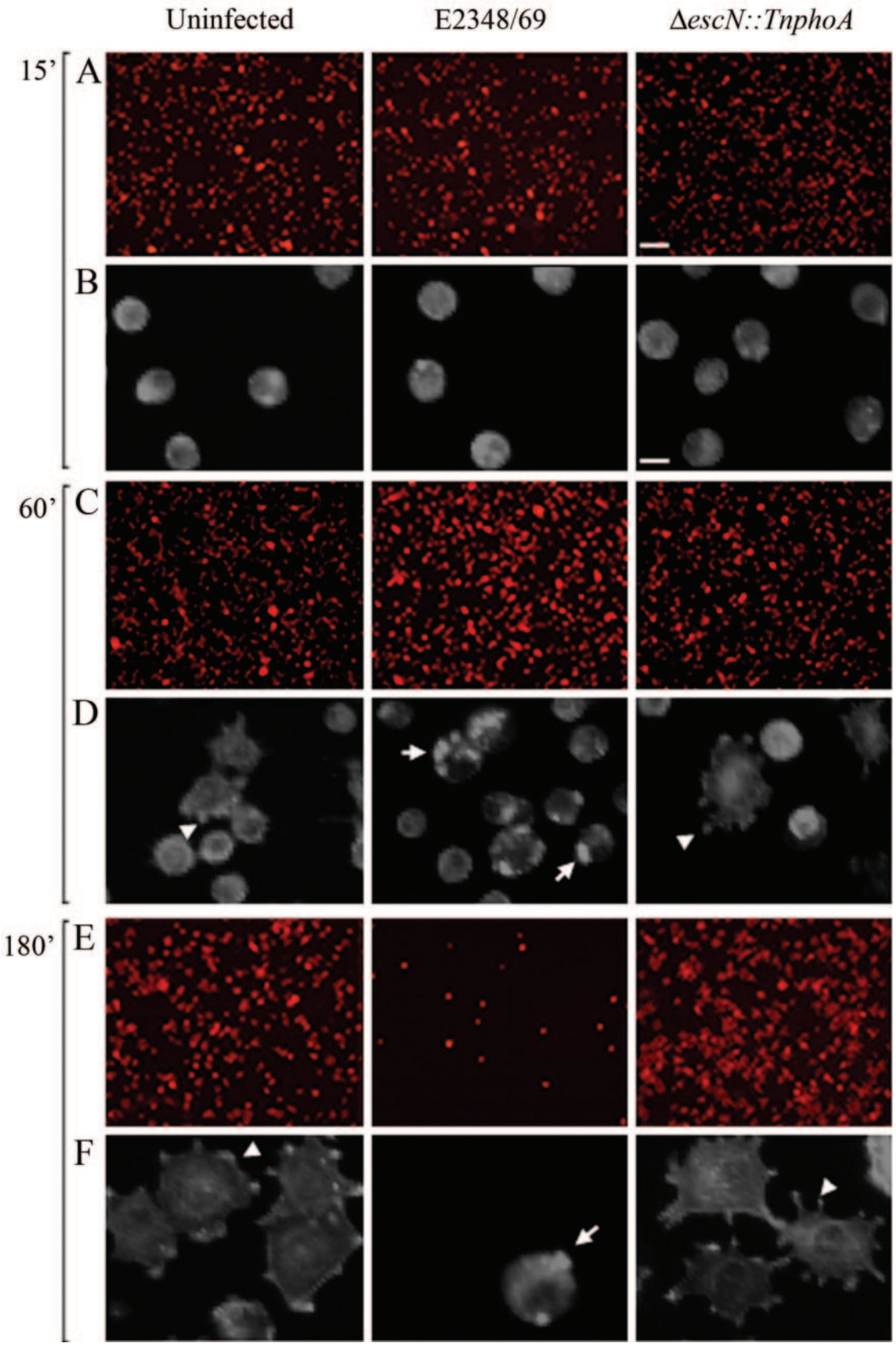


FIG. 1. EPEC uses a TTSS to inhibit fibroblast attachment. Suspended DU17 fibroblasts were infected with preactivated wild-type EPEC or an *escN* mutant or were not infected. Immediately after infection, the cells were seeded into 24-well plates. At 15, 60, and 180 min postinfection, cells were fixed and actin filaments were stained. The micrographs in panels A, C, and E were taken at a low magnification (bar = 200 μm). Higher magnifications are shown in panels B, D, and F (bar = 20 μm). The infecting strains, as well as the infection periods, are indicated. Actin pedestals are indicated by arrows. Filopodia and lamellipodia are indicated by arrowheads.

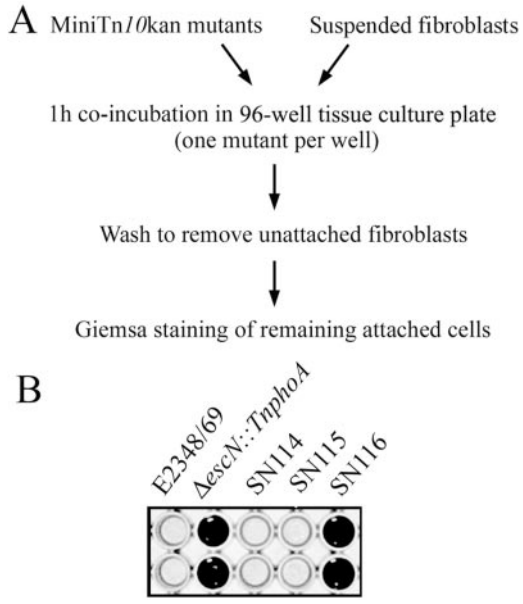


FIG. 2. Isolation of mutants deficient in inhibiting host cell attachment. (A) Flow chart of the screening protocol used for identification of mutants deficient in inhibition of host cell attachment. (B) Part of a 96-well plate showing positive and negative Giemsa staining (wild-type EPEC and *escN* mutant, respectively). Three mutants are also shown; one of these mutants, SN116, is deficient in blocking host cell attachment, while the others act like the wild type. The strains were tested in duplicate.

cultures grown without shaking in LB broth, either at 37°C or at 27°C, or in M9 minimal medium. To create *bvgAS*-repressing conditions (31), 5 mM nicotinic acid or 20 mM $MgSO_4$ was added to the LB broth.

Qualitative and quantitative analysis of actin pedestal formation in infected HeLa cells. HeLa cells were infected, fixed, stained with phalloidin-rhodamine, and analyzed by fluorescence microscopy as previously described (61). To quantify pedestal formation efficacy, the number of HeLa cells with more than four actin pedestals in several randomly chosen fields was divided by the total number of HeLa cells, which yielded the percentage of HeLa cells with actin pedestals. About 100 cells were examined for each strain.

RESULTS

EPEC inhibits cell spreading and ultimately interferes with cell attachment by a TTSS-dependent mechanism. EPEC induces infected host cells to detach from the substratum, and the detachment is particularly enhanced in DU17 fibroblasts (1, 47). We tested whether EPEC also prevents the attachment of suspended host cells to the substratum. Adherent DU17 fibroblasts were trypsinized and infected with activated wild-type EPEC or the EPEC *escN* mutant or not infected. Immediately upon infection, infected cells were seeded, and at different times the unattached cells were washed off. The remaining cells were fixed, actin stained, and examined microscopically (Fig. 1). At 15 min after seeding, infected and uninfected cells were found to be similarly attached. At 60 min after seeding, uninfected cells and cells infected with the *escN* mutant began to spread. Cells infected with wild-type EPEC failed to spread and later (180 min) detached and were washed away (Fig. 1). In contrast, uninfected cells and cells infected

TABLE 3. LEE mutants

Mutant(s)	Gene[a]	Role of associated gene[b]	Mini-Tn*10*kan orientation[c]	Cell attachment (%)[d]	Actin pedestal[e]	EspB secretion[f]
SN6	*cesD*	Chaperone	270	100	−	+/−
SN51	*cesD*	Chaperone	265	100	+/−	+/−
SN97	*escC*	TTSS structure	535	90	−	−
SN5, SN62, SN76, SN90	*escJ*	TTSS structure	188	96, 100, 100, 90	−	−
SN69, SN98	*escJ*	TTSS structure	188	100, 97	−	−
SN99	*escJ*	TTSS structure	399	91	−	−
SN59	*escV*	TTSS structure	229	26	+	+
SN50	*escV*	TTSS structure	238	100	−	−
SN8, SN9	*escV*	TTSS structure	672	95, 100	−	−
SN4	*escV*	TTSS structure	724	81	−	−
SN46	*escV*	TTSS structure	813	91	−	−
SN67	*escD*	TTSS structure	28	50	+	+
SN23	*sepL*	Translocation	192	72	−	−
SN7	*espB*	Translocon	188	100	−	−
SN17	*espB*	Translocon	697	100	−	−
SN115	*escF*	TTSS structure	92	91	−	−

[a] Gene in which mini-Tn*10*kan was inserted.
[b] Known function of the gene in which mini-Tn*10*kan was inserted.
[c] The orientation of the mini-Tn*10*kan cassette in the TTSS gene is indicated by an arrowhead. The number indicates the location within the gene, expressed as number of base pairs from the beginning of the gene.
[d] Percentage of fibroblast reattachment after incubation with mutant bacteria, measured by a protein assay of the remaining fibroblast lysate (100% was the protein level for fibroblast lysate infected by *escN*::*TnphoA*); when there is more than one mutant, the value for each mutant is indicated.
[e] Formation of actin pedestals in HeLa cells infected by the mutant bacteria.
[f] Presence of EspB in bacterial supernatants under TTSS-inducing conditions. +, EspB secretion; −, no EspB secretion; +/−, partial EspB secretion.

with the *escN* mutant remained attached and spread. These findings indicate that wild-type EPEC allows initial host cell attachment to the substratum. However, EPEC ultimately interferes with host cell attachment and spreading in a TTSS-dependent manner. We called this sequence of events "attachment inhibition."

Isolation of mutants deficient in inhibiting cell attachment. Using Giemsa staining in a 96-well format, cell attachment was visible to the naked eye as bluish turbidity at the bottom of a well, whereas a lack of attachment resulted in clear wells (Fig. 2B). We used this assay to screen ~4,000 mini-Tn*10*kan mutants of EPEC for a deficiency in preventing fibroblast attachment (Fig. 2). Mutants that repeatedly failed to prevent host cell attachment were analyzed further by (i) mapping the insertion site, (ii) quantifying the inhibition of cell attachment, (iii) testing EspB secretion, and (iv) microscopically observing the infected HeLa cells. The latter procedure was used to analyze the formation of actin pedestals in infected cells and to determine the pattern of bacterial attachment. We identified 26 different mutants; 21 of these mutants contained insertions located within the LEE, and 5 mutants contained insertions located outside the LEE. The 21 LEE mutants had insertions in *cesD*, *escC*, *escJ*, *escV*, *espD*, *sepL*, *espB*, and *escF* (Table 3).

In most cases, insertion within the LEE genes resulted in mutants deficient in EspB secretion and in inducing actin pedestal formation. These characteristics were also observed for five of the *escV*::mini-Tn*10*kan mutants but not for SN59, which secreted EspB and only partially inhibited cell attachment (26%) (Table 3). The insertion in SN59 was mapped 9 bp upstream of that of SN50, flanking the sequence **C**GTTA TG**C****G** (the boldface C and G are mini-Tn*10*kan insertion sites in SN59 and SN50, respectively, and the underlined methionine codon represents M79). We speculate that in SN59, the mini-Tn*10*kan sequence incidentally forms a ribosomal binding site, which allows translation initiation from M79. This may lead to synthesis of truncated EscV that is sufficient for assembly of a partially active TTSS, which may explain the differences in the *escV* mutant phenotypes.

Another interesting observation was an observation made with the SN67 mutant, which despite its active TTSS, suggested by its EspB secretion and its actin pedestal induction, allowed only partial cell attachment (50%) (Table 3). This may suggest a role for a possible effector; however, the SN67 insertion has been localized to *escD*, which is known to code for a structural protein that together with EscJ forms a ring-like structure in the TTSS inner membrane (30, 50, 51). The insertion in SN67 is located 12 bp upstream of the M14 codon, which may allow translation initiation from M14, as described for SN59. The putative truncated EscD may support the formation of a partially active TTSS. This suggests that high translocation efficiency may be required to block host cell attachment.

Similar phenotypes were observed in SN6 and SN51 mutants, both of which contained insertions in *cesD* encoding a TTSS chaperone for EspD and EspB. These mutants exhibited attenuated TTSS activity and allowed cell attachment, like an *escN* mutant, again suggesting that high translocation efficiency is required to block host cell attachment. In conclusion, aside from mutants with insertions in *cesD* and *escD* all LEE mutants exhibited similar phenotypes, which supports the notion that ultimately EPEC inhibition of host cell spreading and attachment is TTSS dependent. In addition, the results validate our screening methodology.

Five non-LEE mutants were isolated, SN61, SN63, SN64, SN66, and SN116 (Table 4). Strain SN66, in which the insertion was in the *bfpF-P* intergenic region, failed to form microcolonies on infected HeLa cells compared to the wild type (data not shown). Mutants SN61 and SN63 both had an insertion in *micC* encoding a small RNA that negatively regulated the adjacent *ompC* porin (5) (Table 4). In SN64, the mini-Tn*10*kan was localized between two open reading frames, *yehI* and *yehL*, in a truncated gene, similar to the *E. coli* K-12 *yehK* gene. This is a variable region in different *E. coli* isolates, and the role of the genes flanking it has not been determined. The insertion site in SN116 was mapped between *ydeP* and its promoter. Among the non-LEE mutants only SN116 was completely deficient in inhibition of host cell attachment and in EspB secretion. In further studies, therefore, we focused on this mutant.

Overexpression of either *evgA*, *ydeP*, or *ydeO* negatively regulates the production of TTSS and BFP. The mini-Tn*10*kan insertion in SN116 introduced a constitutive promoter (the *kan* promoter) that can direct expression of three downstream genes, *ydeP*, *b1500*, and *ydeO* (Table 4). In *E. coli* K-12 the *ydeP* gene and the *ydeO* promoter are activated upon EvgA overexpression (29, 36). Therefore, we tested the hypothesis that the insertion in SN116 mimics activation of the *evgAS* cascade to downregulate LEE gene expression. To this end, we cloned *evgA*, *ydeP*, *b1500*, or *ydeO* under control of the IPTG-inducible promoter in pSA10. The wild-type EPEC was transformed with the different plasmids, and the production of different LEE proteins was tested in each strain (Fig. 3A). Overexpression of *evgA*, *ydeP*, or *ydeO* caused decreased production of EscJ, EspB, Tir, intimin, and Ler. Overexpression of *ydeP* also repressed BfpA production. In contrast, overexpression of *b1500* affected neither LEE nor BFP protein production (Fig. 3A).

EvgA-mediated repression of LEE genes is not Ler dependent. Ler is a positive regulator of most of the LEE operons (19, 38). Thus, EvgA-mediated *ler* repression (Fig. 3A) might be necessary and sufficient for EvgA-mediated repression of the other LEE genes. The other possibility is that EvgA mediates the repression of LEE promoters, regardless of the presence of Ler. To distinguish between these two options, the wild-type EPEC was transformed with two compatible plasmids, pCNY2441 and pTU14, allowing simultaneous *evgA* and *ler* expression from a *tac* promoter. Expression of LEE proteins was compared in wild-type EPEC, in a strain overexpressing *evgA*, and in a strain overexpressing both *evgA* and *ler*. We found that overexpression of the recombinant Ler did not rescue the *evgA*-mediated repression of Tir and EspB production (Fig. 3B). These results suggest that although EvgA represses *ler* expression, this repression is not required for the EvgA-mediated downregulation of Tir and EspB production.

Search for EvgAS-activating conditions. We hypothesized that under EvgA-activating conditions an EPEC *evgA* mutant should overexpress the TTSS genes. In spite of extensive efforts, we could not define EvgA-activating conditions in EPEC or a TTSS-related phenotype in *evgA*, *ydeP*, *ydeO*, or *ydeP-ydeO* mutants (data not shown). Similarly, neither the environmental conditions that activate EvgA nor a phenotype for the *evgAS*

TABLE 4. Non-LEE mutants

Mutant(s)	Associated gene[a]	Mini-Tn*10*kan orientation[b]	Comment[c]	Cell attachment (%)[d]	Actin pedestal[e]	EspB secretion[f]
SN66	*bfpP*	*bfpE* *bfpF* *bfpP* (117)	Intergenic region insertion	59	+	+
SN61, SN63	*micC*	*ompN* *micC* (47) *ydbK*	Insertion in sRNA	26, 29	+/−	+/−
SN64	*Z3290*	*yehI* *yehK* *yehL* (928)	Unknown function locus	33	+	+
SN116	*ydeP*	*b1502* (58) *ydeP* *b1500* *ydeO*	Intergenic region insertion	100	−	−

[a] Gene closest to the mini-Tn*10*kan insertion.
[b] The orientation of the mini-Tn*10*kan cassette in the associated gene is indicated by an arrowhead. The number indicates either the number of base pairs from the beginning of the gene or the number of base pairs to the ATG of the next downstream gene.
[c] Comment regarding the insertion locus.
[d] Percentage of fibroblast reattachment after incubation with mutant bacteria, measured by a protein assay of the remaining fibroblast lysate (100% was the protein level for fibroblast lysate infected by *escN*::*TnphoA*); when there is more than one mutant, the value for each mutant is indicated.
[e] Formation of actin pedestals in HeLa cells infected by mutant bacteria.
[f] Presence of EspB in bacterial supernatants under TTSS-inducing conditions. +, EspB secretion; −, no EspB secretion; +/−, partial EspB secretion.

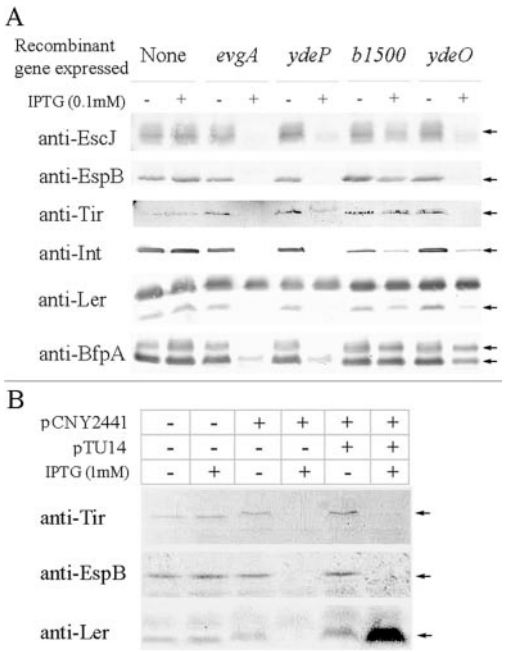
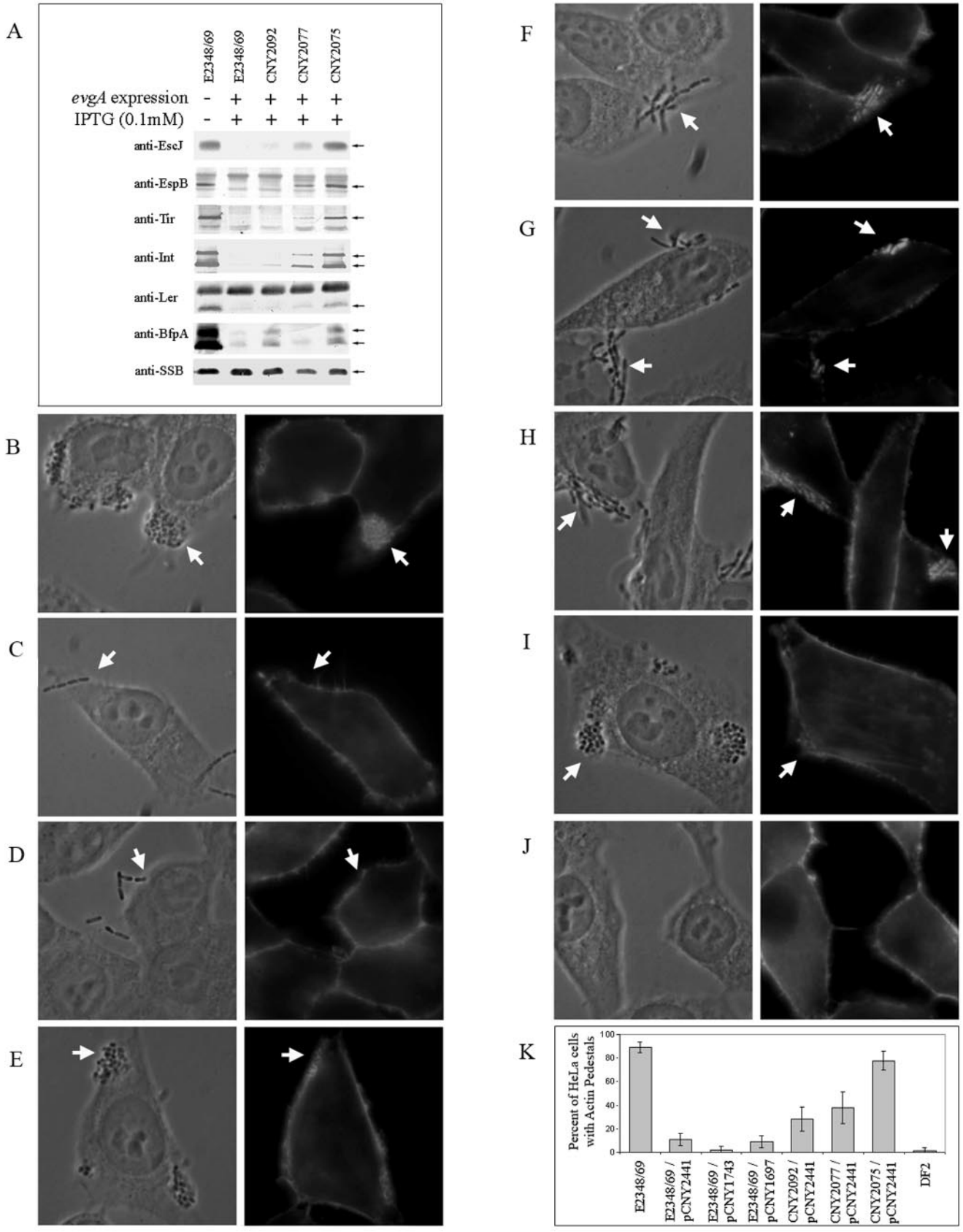


FIG. 3. Overexpression of either *evgA*, *ydeP*, or *ydeO* resulted in decreased production of TTSS and BFP proteins in a Ler-independent pathway. (A) EPEC strains containing expression vectors (pSA10) encoding either *evgA*, *ydeP*, *b1500*, or *ydeO* were grown under TTSS- and BFP-stimulating conditions. Protein extracts were used for immunoblot analysis. The expressed recombinant genes are indicated at the top. IPTG was added as indicated. Expression of *evgA*, *ydeP*, and *ydeO* resulted in decreased expression of EscJ, EspB, Tir, intimin, Ler, and BfpA, as shown by immunoblotting. The corresponding proteins are indicated by arrows. (B) EPEC strains were transformed with pCNY2441 expressing *evgA* and with pTU14 expressing *ler*, as indicated. Cultures were grown under TTSS- and BFP-stimulating conditions, and IPTG was added as indicated. Protein extracts were analyzed by immunoblot analysis with anti-Tir, anti-EspB, and anti-Ler antibodies. EvgA-mediated repression of Tir and EspB production was not affected by *ler* expression. The positions of the corresponding proteins are indicated by arrows.

mutants was found in *E. coli* K-12 (42, 64). The GadXW regulatory cascade was reported to overlap with the regulatory cascade of EvgAS (34) and to indirectly repress the LEE genes (48). Therefore, double mutants, including *evgA*/*gadX*, *evgA*/*gadXW*, *ydeO*/*gadX*, and *ydeO*/*gadXW* mutants, were constructed and tested for differences in expression of TTSS genes under various conditions. However, all of these mutants exhibited a phenotype similar to that of the wild-type strain (data not shown). In conclusion, the environmental conditions that stimulate EvgA activation remain elusive.

EvgA-mediated downregulation of the LEE genes is dependent on the *ydeP-ydeO* region. We next tested whether EvgA-mediated repression of LEE genes is dependent on *ydeP* or *ydeO*. The *evgA*-expressing vector pCNY2441 was introduced into wild-type EPEC and into mutants CNY2092, CNY2077, and CNY2075 (Δ*ydeP*::*kan*, Δ*ydeO*::*kan*, and Δ*ydePO*::*kan*, respectively). According to an immunoblot analysis, *evgA* expression resulted in repression of EscJ, EspB, Tir, intimin, Ler, and BfpA production in the wild-type strain but not in the Δ*ydePO*::*kan* mutant. Expression of *evgA* in the Δ*ydeP*::*kan* mutant or in the Δ*ydeO*::*kan* mutant caused only partial repression of the production of LEE proteins (Fig. 4A). As a negative control, we examined the production of single-strand-binding protein (SSB), a housekeeping protein. SSB expression was not altered in the various strains, indicating that there was a specific effect. These results suggest that EvgA activates the expression of both *ydeP* and *ydeO*, which are required for full efficiency of LEE repression.

To substantiate these results, we tested the abilities of the different strains to induce the formation of actin pedestals in infected HeLa cells (Fig. 4B to K). In these experiments, we used wild-type EPEC and a *ler* mutant as positive and negative controls, respectively (Fig. 4B, I, and K). Overexpression of recombinant *evgA*, *ydeP*, or *ydeO* caused a reduction in the efficiency of actin pedestal formation (Fig. 4C to E and K). However, actin pedestal formation was evident when *evgA* was expressed in the Δ*ydeP*::*kan* and Δ*ydeO*::*kan* mutants, and deletion of the entire *ydeP-ydeO* region (in the Δ*ydePO*::*kan*


A
E2348/69
E2348/69
CNY2092
CNY2077
CNY2075
evgA expression - + + + +
IPTG (0.1mM) - + + + +
anti-EscJ
anti-EspB
anti-Tir
anti-Int
anti-Ler
anti-BfpA
anti-SSB
B
C
D
E
F
G
H
I
J
K
Percent of HeLa cells with Actin Pedestals
100
80
60
40
20
0
E2348/69
E2348/69 / pCNY2441
E2348/69 / pCNY1743
E2348/69 / pCNY1697
CNY2092 / pCNY2441
CNY2077 / pCNY2441
CNY2075 / pCNY2441
DF2

mutant) completely suppressed the ability of EvgA to repress the formation of actin pedestals (Fig. 4F to H and K).

DISCUSSION

EPEC caused host cell detachment in a TTSS-dependent manner (47). Mutants with mutations in the TTSS effector genes (*tir*, *map*, *espH*, and *espF*) still induce host cell detachment (47; unpublished results). Using a different approach, we found here that although wild-type EPEC allows initial attachment of suspended fibroblasts, EPEC ultimately interferes with host cell spreading and attachment in a TTSS-dependent manner. Presumably, EPEC injects a putative effector(s), which may disrupt the focal adhesion, blocking cell spreading (47). A screening method to identify mutants with insertions in the gene(s) coding for the putative effector(s) was designed and used. We expected that inactivation of the putative effector gene would cause a reduction in the inhibition of host cell spreading and attachment. However, such mutants were not identified upon screening. The mutagenesis method that we used resulted in some "hot spots," where several insertions were found in the same gene. Future analyses may combine the use of several transposons, which would result in more randomized mutagenesis. It is also possible that we could not identify the putative effector because EPEC encodes more than one effector capable of mediating inhibition of host cell attachment.

Nevertheless, mutants with insertions in other genes were recovered, and most of the genes were localized in the LEE. These insertions fully or partially inactivated the TTSS. The mutations of the few non-LEE mutants were localized to genes that included *micC* and were upstream of *bfpP*, *yehL*, and *ydeP*. Among these mutants, only the mutant with an insertion upstream of *ydeP* was completely deficient in inhibition of host cell attachment and was therefore analyzed further. The insertion upstream of *bfpP* probably interfered with biogenesis of the BFP, which is required for efficient host cell infection (4, 13). Furthermore, the BFP mediates bacterium-bacterium interactions (12, 24), which may be necessary for inducing host cell detachment. The insertion upstream of *ydeP* was associated with reduced inhibition of cell attachment. The *ydeP* promoter is positively regulated by EvgAS (36), which is highly homologous to BvgAS (57), which is known to regulate *Bordetella pertussis* virulence. Recently, *evgS* and *ydeP* mutants of EHEC serotype O26:H- were found to be attenuated in colonization of calf intestines (58). We thus focused on the role of the EvgAS cascade in regulating production of the LEE and BFP proteins.

Overexpression of the unphosphorylated response regulator mimics two-component system activation, resulting in altered expression of the target genes in the absence of the environmental signals responsible for their phosphorylation (41). This strategy, together with microarray analysis of *E. coli* K-12, was used to identify five operons that include 15 genes that are directly activated by EvgA. Among these genes is *ydeO*, which encodes a member of the AraC/XylS family of transcriptional regulators (20, 36). YdeO further activates, directly or indirectly, expression of an additional 18 genes (36), including *gadE* (*yhiE*), encoding a LuxR family transcriptional activator (33), and *yhiF*, encoding a DNA-binding response regulator homolog, which negatively regulates several LEE operons in EHEC O157 (52). Our findings show that *evgA* overexpression in EPEC represses both the TTSS genes and the BFP genes. The *evgA*-mediated TTSS and BFP repression is fully suppressed in a strain with a deletion in the *ydePO* region. In addition, overexpression of *ydeO* and *ydeP* is sufficient to cause repression. These results indicate that *evgA*-mediated repression is dependent on YdeO and YdeP. These proteins might act indirectly, perhaps by inducing production of YhiF and/or YhiE (52). Interestingly, we found that EvgA-mediated repression of *ler* is not required for repression of the other LEE genes.

A spontaneous, constitutive, active *evgS* mutant (29) phosphorylates and activates EvgA (14). In addition, a highly specific interaction has been found between the histidine-containing phosphotransfer module of EvgS and EvgA, and their intermolecular phosphorelay has been found to be necessary for EvgA activation (44). Assuming that EvgA is activated upon EvgS stimulation, we predicted that under EvgS-stimulating conditions, the TTSS genes should be repressed in a wild-type EPEC but not in an *evgA* mutant. However, our comparative study of the EPEC *evgA* mutant and the wild-type strains under various conditions, including *bvgAS*-repressing conditions, did not reveal any phenotype. Therefore, the physiological conditions necessary for activation of the EvgS kinase in EPEC remain unknown. Similarly, the nature of the EvgAS activation conditions in *E. coli* K-12 also remains obscure (42, 64).

FIG. 4. *evgA*-mediated TTSS repression is dependent on intact *ydeP* and *ydeO* genes. (A) Production of EscJ, EspB, Tir, intimin, and Ler examined in different strains containing (+) or not containing (−) pCNY2441 (*evgA*). IPTG was added as indicated. In the *ydeP* mutant (CNY2092), TTSS expression remained repressed, but BfpA expression was restored. In the *ydeO* mutant (CNY2077), BfpA expression remained repressed, but TTSS expression was restored. In the *ydeP-ydeO* mutant (CNY2075), TTSS expression was restored, and expression of BfpA was partially restored. SSB expression, used as control, was similar in the various strains. The positions of the corresponding proteins are indicated by arrows. (B to J) HeLa cells infected by various strains examined by phase-contrast microscopy (left column) and fluorescent microscopy (right column). The locations of some bacteria are indicated by arrows. Wild-type EPEC formed microcolonies on the host cell surface and induced the formation of actin pedestals (B). In panels C, D, and E, cells were infected with EPEC containing pCNY2441 (*evgA*) (C), pCNY1743 (*ydeP*) (D), or pCNY1697 (*ydeO*) (E). Overexpression of *ydeO*, *evgA*, or *ydeP* resulted in reduced pedestal formation (C to E). In panels F, G, and H cells were infected with various mutated strains, including CNY2092 (Δ*ydeP*::*kan*) (F), CNY2077 (Δ*ydeO*::*kan*) (G), and CNY2075 (Δ*ydePO*::*kan*) (H), all overexpressing *evgA* from pCNY2441. Cells that were infected with DF2 (Δ*ler*::*kan*) (I) or were not infected (J) served as negative controls. In panel K pedestal formation efficacy is shown. Reduced pedestal formation was evident in cells infected with wild-type EPEC expressing *evgA* [E2348/69(pCNY2441)], *ydeP* [E2348/69(pCNY1743)], or *ydeO* [E2348/69(pCNY1697)]. However, pedestal formation was restored or partially restored upon *evgA* overexpression in CNY2092 (Δ*ydeP*::*kan*), CNY2077 (Δ*ydeO*::*kan*), or CNY2075 (Δ*ydePO*::*kan*). Infection with the wild type (E2348/69) and infection with a *ler* mutant (DF2) served as positive and negative controls, respectively. Standard errors are indicated by vertical bars.

An alternative explanation for the lack of a phenotype for the *evgA* mutant is that the physiological function of the EvgAS system is redundant with respect to other regulatory cascades. Indeed, previous reports showed that there is a partial overlap between the EvgAS and GadXW cascades. This overlap includes activation of YhiE (GadE) and YhiF (36, 55). Moreover, GadX overexpression in EPEC represses the expression of TTSS and BFP genes via expression of the *perABC* operon (48). The reports mentioned above and the lack of a phenotype in the EPEC *evgA* mutant led us to construct double mutants with mutations in *evgA* or *ydeO* and in *gadX* or the *gadX-gadW* region. However, the four double mutants examined did not differ from the wild type.

In conclusion, many LEE regulators, most of which function at the transcriptional level, have been discovered. Here we report that the EvgAS two-component system, apparently activated by an unknown external signal, represses TTSS expression. Identification of the nature of the elusive EvgS activating signal should elucidate the physiological role of EvgAS-mediated repression of the TTSS genes in EPEC.

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