Supporting Information

Alsharif et al. 10.1073/pnas.1422986112

SI Materials and Methods

Strains, Cell Lines, and Growth Conditions. Bacteria were maintained on MacConkey agar and, unless otherwise stated in the figure legends, subcultured for experiments in LB at 37 °C shaking. Where required for selection, antibiotics were added to the medium (35 μg/mL tetracycline, 35 μg/mL chloramphenicol, 200 μg/mL ampicillin). HeLa and Caco-2 epithelial cell lines were cultured at 37 °C and under 5% (vol/vol) CO_2 in DMEM containing 10% (vol/vol) heat-inactivated FBS, 4,500 mg/L glucose, 0.5 mM L-glutamine, 100 U/mL penicillin, and 20 μg/mL streptomycin.

Infection of Host Cells under Static and Flow Conditions. Tissue culture cells were washed with PBS before the addition of bacteria in tissue culture medium without antibiotics. Bacteria were added to give a MOI of 10 before incubation at 37 °C for 30 min to 4 h, depending on the experiment (see figure legends for details). For enumeration of bacteria, samples were removed at points, as indicated, and were serially diluted, plated on LB agar plates, and incubated at 37 °C for 16 h, and cfu were determined. For enumeration of host-adherent bacteria, host cells were washed three times with PBS and lysed with PBS containing 1% Triton X-100 before dilution plating. For flow experiments, host cells were cultured in flow cells 1 d before infection. To infect, EHEC were introduced onto the host cell layer, the flow was discontinued, and flow cells were left at 37 °C for 1 h under static conditions. Fresh DMEM was then flowed across the cell layers at variable flow rates, resulting in shear forces from 0 to 10 dynes/cm^2. Flow cells were then either perfused with 3.2% (wt/vol) paraformaldehyde to fix samples before imaging or with PBS+1% Triton X-100 to harvest samples for plating and β-galactosidase assays, as described later.

Imaging of EHEC Infections. For microscopy, samples were fixed with 3.2% (wt/vol) formaldehyde, permeabilized with 0.1% Triton X-100, and stained for 10 min with rhodamine-phalloidin to visualize F-actin and Hoechst to visualize DNA. Samples were mounted using ProLong Gold Antifade Mountant, and images were captured on a Nikon Eclipse Ti fluorescence microscope and analyzed and prepared for publication using Image J and Corel Draw ×5.

1. Hartland EL, et al. (1999) Binding of intimin from enteropathogenic Escherichia coli to Tir and to host cells. *Mol Microbiol* 32(1):151–158.

Surface Coating with Pure Substrates for Bacterial Adhesion. Coverslips and flow cell surfaces were coated with poly-L-lysine, Tir peptide, or α-LPS antibody to enable bacterial attachment independent of host cells. For poly-L-lysin coating, surfaces were incubated with poly-L-lysin (0.2 mg/mL aqueous solution) for 1 h at 22 °C. Solution was aspirated and surface left to dry for 1 h at 37 °C. Surface was rinsed with PBS before bacterial attachment. For coating with Tir peptide, His-Tir-M was prepared as described previously (1), adjusted to 10 μg/mL in PBS, and incubated with the surface overnight at 4 °C. For coating with α-LPS antibody, antibody P3C6 (ab75244, specific against *E. coli* O157:H7 O-antigen) was adjusted to 10 μg/mL in PBS and incubated with the surface overnight at 4 °C. Peptide or antibody was removed, and the surface was rinsed with PBS before the experiment.

Measurement of β-Galactosidase Activity and Relative Transcriptional Activity. Promoter induction of *lacZ* transcriptional reporters was measured by assaying EHEC strains for β-galactosidase activity. EHEC reporter strains were grown either in planktonic LB or DMEM cultures at 37 °C shaking at 200 rpm to an OD_{600} of ∼0.5. Alternatively, bacteria for the assay were sampled from the supernatant of infected host cells grown in DMEM. Host-adherent bacteria were recovered after removing culture supernatants, washing host cells with PBS three times, and host cell lysis in PBS+1% Triton X-100. β-galactosidase activity was measured using the Miller method and is shown in Miller Units for planktonic cultures. Where samples taken from infection experiments were compared, β-galactosidase activities were expressed in terms of bacterial numbers (cfu/mL) instead of OD_{600}, and are thus expressed as "relative transcriptional activity" instead of Miller Units.

Fluorescence Plate Assays. EHEC strains in DMEM were introduced either into empty 96-well plates or plates containing HeLa cells at 150,000 cells/mL Plates were incubated at 37 °C, and whole-well fluorescence was measured on a BMG Labtech Omega microplate reader (485–512 nm bandpass filter for excitation and 460–10 nm bandpass filter for emission) at 1, 2, 3, or 4 h. Each sample was measured in triplicate wells, and at least three independent experiments were performed.

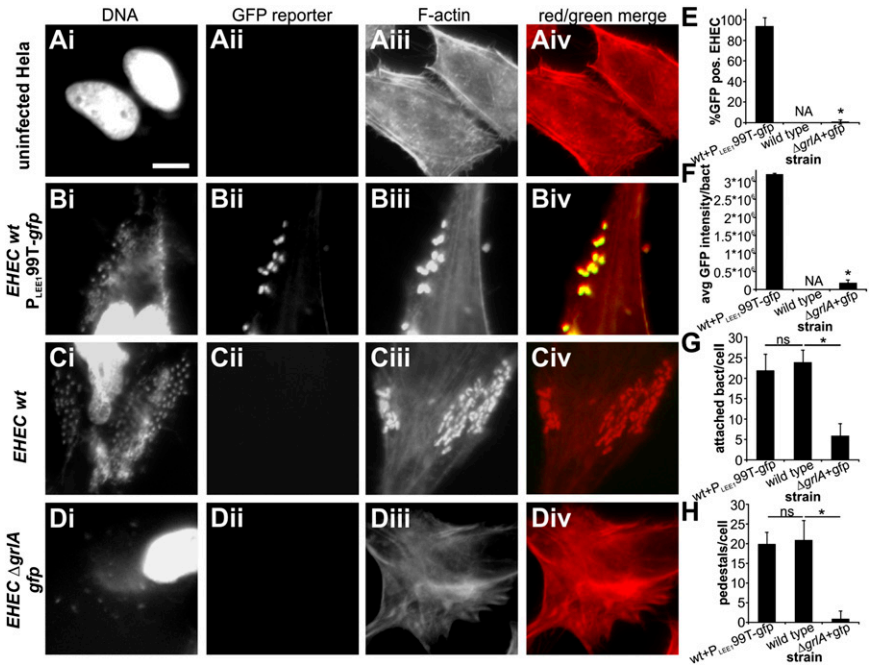

Fig. S1. Phenotype and LEE1 promoter induction in EHEC reporter strains infecting HeLa cells. HeLa cells were either left uninfected (*A*) or infected with EHEC wild-type containing P_{LEE1}99T-*gfp* (*B*), EHEC wild-type (*C*), or EHEC Δ*grlA* harboring a promoter-less *gfp* reporter (*D*), at an MOI 10 for 4 h. Samples were fixed and DNA (Hoechst), reporter activation (GFP), and F-actin (rhodamine-phalloidin) were visualized by fluorescence microscopy. (Scale bar, 10 μm.) Percentage GFP-positive bacteria (*E*), average GFP intensity per bacterium (for GFP-positive cells) (*F*), number of attached bacteria per host cell (*G*), and number of pedestals per host cell (*H*) were determined from these experiments. Data are representative of three independent experiments (>100 HeLa cells each). The asterisk denotes significant differences between samples based on Student's *t* test ($P < 0.05$). ns, not significant ($P \geq 0.05$). NA, not analyzed (fluorescence in reporter-less wild-type EHEC).

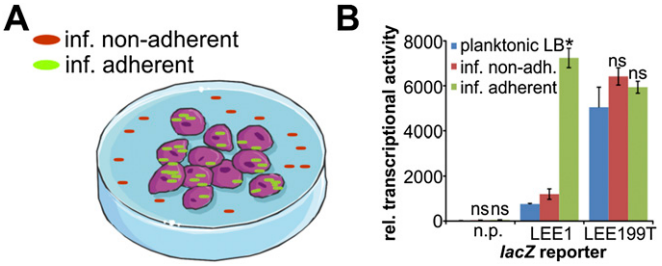

Fig. S2. Measurement of LEE1 induction in EHEC wild-type bacteria during infection. Schematic depicting the experiment measuring LEE1 promoter activity in nonadherent and host-adherent bacteria (*A*). Host cells adhere to the culture vessel and are infected with EHEC reporter strains (MOI 10, 4 h). Subsequently, nonadherent bacteria (red) were recovered from the supernatant. Host cells were then washed and Triton-X100 lysed to recover adherent bacteria (green). Both fractions were used to determine β-galactosidase activity and cfu/mL. (*B*) β-galactosidase activity was normalized to bacterial counts and is shown as relative transcriptional activity of EHEC wild-type bacteria harboring a promoter-less *lacZ* reporter (n.p.), inducible P_{LEE1}-*lacZ*, or constitutively active P_{LEE1}99T-*lacZ*. Values were compared with those from bacteria grown in planktonic LB cultures (blue). The asterisk denotes significant differences between nonadherent and adherent fractions based on Student's *t* test ($P < 0.05$; $n = 3$). ns, not significant ($P \geq 0.05$).

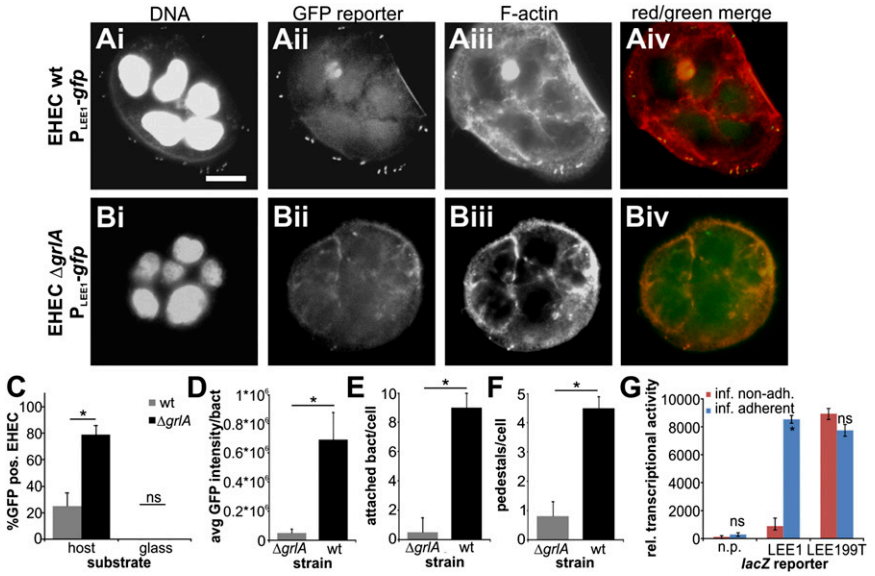

Fig. S3. Phenotype and LEE1 promoter induction in EHEC reporter strains infecting Caco-2 cells. EHEC wild-type (*A*) or EHEC Δ*grlA* (*B*) harboring a P_{LEE1}-*gfp* transcriptional fusion as reporter were used to infect Caco-2 cells (MOI 10, 4 h). Samples were fixed and DNA (Hoechst), reporter activation (GFP), and F-actin (rhodamine-phalloidin) were visualized by fluorescence microscopy. (Scale bar, 20 μm.) Percentage GFP-positive bacteria (*C*), average GFP intensity per bacterium (for GFP-positive cells) (*D*), number of attached bacteria per host cell (*E*), and number of pedestals per host cell (*F*) were determined from these experiments. Data are representative of three independent experiments (>100 Caco cells each). LEE1 induction was also determined using EHEC wild-type bacteria containing a promoter-less *lacZ* reporter (n.p.), inducible P_{LEE1}-*lacZ*, or constitutively active P_{LEE1}99T-*lacZ*. Caco-2 cells were infected with these strains (MOI 10, 4 h), nonadherent (red) and host-adherent (blue) bacteria separated and β-galactosidase activity determined in each of these fractions and expressed as a function of bacterial counts to give relative transcriptional activities (*G*). The asterisk denotes significant differences between nonadherent and adherent fractions based on Student's *t* test ($P < 0.05$, $n = 3$). ns, not significant ($P \geq 0.05$).

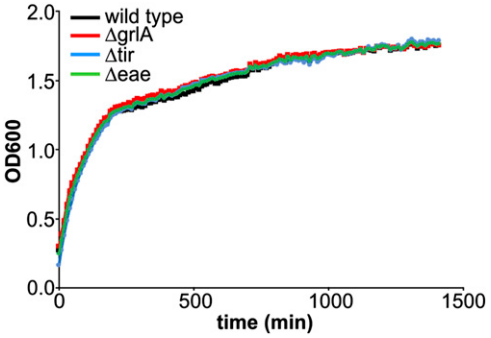

Fig. S4. Growth of EHEC wild-type and deletion mutants. EHEC wild-type or deletion strains were grown overnight in LB broth and diluted into DMEM to give an initial OD_{600} of 0.25. Strains were then grown in a 96-well plate at 37 °C under intermittent shaking and OD_{600} was measured every 10 min over 23.5 h. Data are representative of three independent experiments done in triplicate.

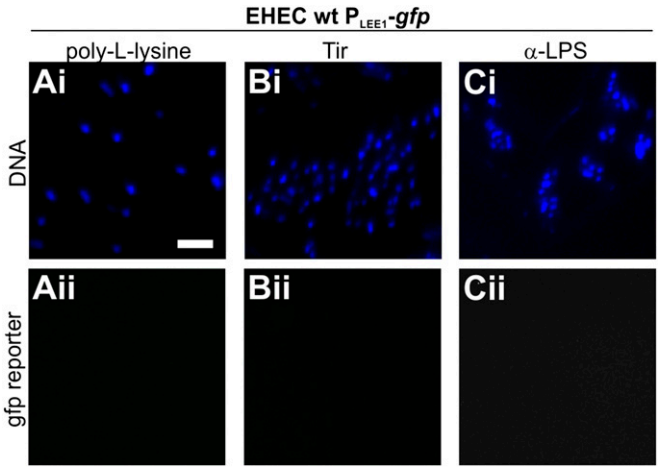

Fig. S5. Bacterial attachment to soluble substrates does not cause LEE1 induction. EHEC wild-type strain containing a P$_{LEE1}$-*gfp* reporter was incubated with soluble substrates: poly-L-lysine (*A*), Tir-peptide (*B*), or α-LPS antibody (*C*) under static conditions for 4 h before imaging bacteria by DNA staining (Hoechst, *Top*) and LEE1 activity by GFP fluorescence (*Bottom*). (Scale bar, 5 μm.)

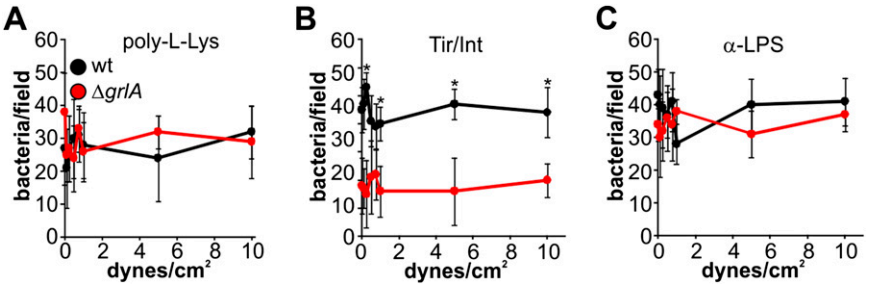

Fig. S6. The number of substrate-attached bacteria is independent of fluid shear force. The total number of attached bacteria per field was enumerated for both EHEC wild-type (black) and Δ*grlA* (red) strains and for channels coated with poly-L-lysine (*A*), Tir-peptide (*B*), or α-LPS antibody (*C*). In each case, the total number of bacteria remained constant with increasing fluid shear force between 0 and 10 dynes/cm^2. Data are representative of three independent experiments (>100 cells each). The asterisk denotes significant differences between samples based on Student's *t* test ($P < 0.05$).

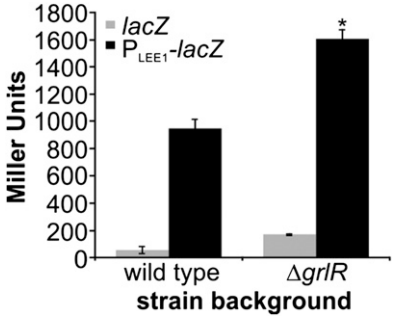

Fig. S7. Effect of *grlR* deletion in EHEC on LEE1 induction. LEE1 promoter activity was monitored using either promoterless *lacZ* (gray) or P$_{LEE1}$-*lacZ* (black) transcriptional fusion constructs in EHEC wild-type or Δ*grlR* cells grown in DMEM to an OD_{600} of ~0.5 at 37 °C. Data are representative of three independent experiments, and the asterisk denotes significant differences between wild-type and Δ*grlR* backgrounds, based on Student's *t* test ($P < 0.05$).

Table S1. Strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
EHEC wild-type	EHEC O157:H7 Sakai 813 (lacking Shiga toxins)	Gift from S. Sasakawa
EHEC Δ*tir*	EHEC O157:H7 Sakai 813 *Δstx Δtir*	This study
EHEC Δ*eae*	EHEC O157:H7 Sakai 813 *Δstx Δeae* (intimin)	This study
EHEC Δ*grlA*	EHEC O157:H7 Sakai 813 *Δstx ΔgrlA*	1
EHEC Δ*grlR*	EHEC O157:H7 Sakai 813 *Δstx ΔgrlR*	1
Plasmids		
pRW50	Low copy number plasmid; encodes for tetracycline resistance; carries multiple cloning sites that allow cloning of a promoter fragment, which then controls the expression from *lacZ* as a transcriptional fusion	2
pRW224/U9 (promoterless *lacZ*)	Low copy number plasmid derived from pRW50 that lacks *trpAB* genes; encodes for tetracycline resistance; allows cloning of a promoter fragment that controls the expression from *lacZ* as a transcriptional fusion	1
pRW224/LEE10-568 (P_{LEE1}-*lacZ*)	A derivative of pRW224 carrying an EcoRI-*Hind*III LEE1 promoter (position −568 to position −19 relative to the Ler translation start site) as a transcriptional fusion to *lac*Z	1
pRW224/LEE20-203 99T (P_{LEE1}99T-*lacZ*)	A derivative of P_{LEE1}-*lacZ* carrying an *Eco*RI-*Hind*III fragment (position −203 to position 158 relative to the Ler translation start site) as a transcription fusion to *lacZ*	1
pRW400	Low copy number plasmid derived from pRW224 that carries a *gfp* gene and encodes for tetracycline resistance	This study
pRW400/U9 (promoterless *gfp*)	A derivative of pRW224/U9 where *lacZ*, *lacY*, and *lacA* genes were replaced with *gfp* in frame downstream of the multiple cloning site	This study
pRW400/LEE100 (P_{LEE1}-*gfp*)	A derivative of pRW400/U9 that carries *LEE*100 promoter between EcoRI-HindIII sites as a transcription fusion of *gfp*	This study
pRW400/LEE99T (P_{LEE1}99T-*gfp*)	A derivative of *LEE*100/pRW400 that carried *LEE*107.199T between EcoRI-HindIII sites as a transcription fusion of *gfp*	This study
pACYC184	A cloning vector used to clone gene fragments under the control of their own promoter and encodes for chloramphenicol and tetracycline resistance	3
pSI01 (pGrlRA)	A derivative of pACYC184 carrying the *grlRA* operon, including its promoter region cloned into HindIII and *Sa*lI sites	1
pSI02 (pGrlA)	A derivative of pSI01 carrying a *grlR* deletion	1
pSI03 (pGrlR)	A derivative of pSI01 carrying a *grlA* deletion	1

1. Islam MS, Bingle LE, Pallen MJ, Busby SJ (2011) Organization of the LEE1 operon regulatory region of enterohaemorrhagic Escherichia coli O157:H7 and activation by GrlA. *Mol Microbiol* 79(2):468–483.
2. Lodge J, Williams R, Bell A, Chan B, Busby S (1990) Comparison of promoter activities in Escherichia coli and Pseudomonas aeruginosa: Use of a new broad-host-range promoter-probe plasmid. *FEMS Microbiol Lett* 55(1-2):221–225.
3. Chang AC, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* 134(3):1141–1156.