Host attachment and fluid shear are integrated into a mechanical signal regulating virulence in Escherichia coli O157:H7

Alsharif, Ghadah; Ahmad, Sadia; Islam, Md Shahidul; Shah, Riddhi; Busby, Stephen J; Krachler, Anne Marie

DOI:
10.1073/pnas.1422986112

Document Version
Peer reviewed version

Citation for published version (Harvard):
Alsharif, G, Ahmad, S, Islam, MS, Shah, R, Busby, SJ & Krachler, AM 2015, 'Host attachment and fluid shear are integrated into a mechanical signal regulating virulence in Escherichia coli O157:H7', *National Academy of Sciences. Proceedings*, vol. 112, no. 17, pp. 5503-5508. https://doi.org/10.1073/pnas.1422986112

Host attachment and fluid shear are integrated into a mechanical signal regulating virulence in *Escherichia coli* O157:H7

Ghadah Alsharif[1], Sadia Ahmad[1], Md. Shahidul Islam[2], Riddhi Shah[1], Stephen J W Busby[1], Anne Marie Krachler[1,*]

[1]Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, B15 2TT Birmingham, UK

[2]Department of Biotechnology, Bangladesh Agricultural University, BAU Main Road, Mymensingh 2202, Bangladesh

[*]Correspondence to: a.krachler@bham.ac.uk

Classification: Biological Sciences; Microbiology

Keywords: enterohemorrhagic *Escherichia coli*, locus of enterocyte effacement, attaching/effacing pathogens, gastrointestinal infection, mechanosensing, host-pathogen interactions

ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) is a food-borne pathogen causing hemorrhagic colitis and hemolytic uremic syndrome. EHEC colonize the intestinal tract, through a range of virulence factors encoded by the locus of enterocyte effacement (LEE) as well as Shiga toxin. Although the factors involved in colonization and disease are well characterized, how EHEC regulates their expression in response to a host encounter is not well understood. Here, we report that EHEC perceives attachment to host cells as a mechanical cue that leads to expression of LEE-encoded virulence genes. This signal is transduced via the LEE-encoded global regulator of Ler, GrlA, and further enhanced by levels of shear force similar to peristaltic forces in the intestinal tract. Our data suggests that, in addition to a range of chemical environmental signals, EHEC is capable of sensing and responding to mechanical cues in order to adapt to its host's physiology.

SIGNIFICANCE

Enterohemorrhagic *Escherichia coli* (EHEC) is a food-born pathogen. It can cause bloody diarrhea and hemolytic uremic syndrome, which can lead to severe clinical complications such as kidney failure. The main factors triggering disease are well known and include type III secreted effectors, adhesins and Shiga toxins. Much less is known about how these factors are induced in response to the environmental transition that bacteria experience during transfer into and passage through the host. We show here that while positive regulators of virulence are induced during passage through the host, they are only activated to increase virulence as a result of force generated by host cell contact. Thus, mechanosensation is a way of integrating multifactorial environmental cues to fine-tune virulence regulation.

\body

INTRODUCTION

Pathogens frequently undergo drastic environmental transitions as a direct result of their transmission between different environmental and host niches. In doing so, their gene expression patterns dramatically change to achieve niche adaptation and ensure energy efficiency necessary for survival. Individual cues causing such environmental switches are generally well understood across a range of pathogenic organisms. How integration of such multifactorial cues and, as a result, robust regulation of virulence in response to a range of different hosts is achieved and has evolved is much less understood.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a food-borne pathogen and important cause of bloody diarrhea worldwide (1). In some cases, EHEC infection can lead to hemolytic uremic syndrome and severe clinical complications, including kidney failure. EHEC can persist in environmental niches, as well as colonize the gastrointestinal tract of ruminants and human hosts. Virulence factors contributing to intestinal colonization and establishment of disease in humans are well characterized and include type III secreted effector proteins, factors mediating intimate adhesion (Tir/Intimin) and Shiga toxins. Factors implicated in the formation of attaching and effacing (A/E) lesions, which leads to the loss of microvilli from the intestinal brush border and as a result severe diarrhea, include the Type III secretion system (T3SS) as well as Tir and Intimin (2, 3). These are encoded by a pathogenicity island termed locus of enterocyte effacement (LEE) consisting of five major transcriptional units, LEE1-5 (4). All five units are subject to shared regulation by Ler (LEE encoded regulator), the master regulator of LEE and of other, non-LEE encoded virulence factors (5). This genetic organization is conserved across other A/E pathogens, including enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium* (6, 7). Ler is encoded in the first transcriptional unit of LEE, LEE1, and works mainly by antagonizing global gene repression imposed by H-NS (8). Regulation of Ler is responsive to many environmental cues reflective of the transition in lifestyle as a result of uptake by and passage through the host. These include changes in metabolites, CO_2 concentration and the presence of host immune effectors and adrenal hormones, amongst others (9-12). Many of these cues directly converge on Ler, while others require the global regulator of Ler (GrlA), a LEE encoded positive regulator of Ler expression, but all result in global regulation of LEE-encoded genes and thus

virulence (13-15). However, it is not known how these multifactorial environmental cues are integrated to achieve a spatially and temporally coordinated response to the presence of the host tissue. Here, we describe how initial attachment to host cells generates a mechanical cue, which is further enhanced by fluid shear levels present in the host intestinal tract and is required to fully activate Ler and thus LEE-encoded virulence mechanisms, in a GrlA-dependent manner. Our data suggests that, in addition to a range of chemical signals, EHEC is capable of directly sensing and responding to mechanical cues in order to adapt to its host's physiology and fine-tune virulence activation. In light of recently published data demonstrating mechanosensation as a regulatory cue inducing *Pseudomonas aeruginosa* virulence, this study highlights a remarkable case of parallel evolution, where functionally distinct pathogens have integrated mechanosensation as a basic physical mechanism into their regulatory circuitry to achieve control of virulence pathways (16).

RESULTS

Attachment to host cells triggers LEE induction in a GrlA-dependent manner. LEE1 is the first transcriptional unit within the LEE region and encodes Ler, the master regulator of EHEC virulence gene expression. Previous reports show only a moderate induction of LEE1 promoter activity upon exposure to individual environmental cues, but many of these studies were done in *E. coli* K12 as a surrogate strain, thus eliminating many EHEC-specific factors relevant to virulence regulation (15, 17). Others were done in EHEC strains, but not in the context of host cells (18). In this study, we set out to investigate the direct effects of host cell attachment on LEE-encoded virulence gene regulation in the EHEC strain Sakai 813, a Shiga-toxin negative derivative of the original Sakai isolate. We analyzed LEE1 promoter (P_{LEE1}) activity, using EHEC reporter strains transformed with either P_{LEE1}-*lacZ* or P_{LEE1}-*gfp* transcriptional fusions, upon contact with host cells. We infected Hela epithelial cells with EHEC for four hours and first analyzed LEE1 promoter induction and infection phenotype in situ, using fluorescence microscopy of P_{LEE1}-*gfp* reporter strains. Wild type bacteria efficiently attached to Hela cells and formed actin pedestals, apparent from FAS test, as previously described (Figure 1A), (19). Most host-attached bacteria also showed strong LEE1 promoter activation. Strikingly, bacteria adsorbed to the glass slide rather than attached to host cells, showed no or low GFP fluorescence,

indicating that *ler* induction is enhanced upon attachment to host cells compared to exposure to DMEM alone, which has previously been described as a cue for *ler* activation (Figure 1C), (17). Since GrlA is a LEE-encoded activator of *ler*, and thus the entire LEE region, we also tested LEE1 promoter activation in a *ΔgrlA* background. In contrast to wild type bacteria, LEE1 promoter activity remained low in a *ΔgrlA* background, even in bacteria attached to host cells (Figure 1B-D). Lower LEE1 promoter induction, and thus lower activation of the entire LEE region in the *ΔgrlA* background, was also apparent from the infection phenotype – both the number of attached bacteria per host cell, and the bacteria's ability to form actin pedestals was significantly decreased (Figure 1E, F). Introduction of the different extrachromosomal transcriptional reporters did not, in itself, alter the bacteria's ability to attach or form pedestals – both EHEC wild type and wild type containing a previously described, constitutively active P_{LEE1}-*gfp* fusion (P_{LEE1}99T-*gfp*) showed similar levels of attachment and pedestal formation (Figure S1), (17).

We also tested LEE1 promoter induction in EHEC strains transformed with P_{LEE1}-*lacZ* transcription fusions. β-galactosidase activity was measured in host-attached or non-attached reporter strains isolated from infected host cell cultures and normalized to bacterial counts determined from these samples (Figure S2A). Exposure to DMEM (the cue experienced by non-adherent bacteria isolated from infected cultures) resulted in a moderate increase in *ler* induction, which is in agreement with previous findings (17). Host-adherent bacteria, in contrast, showed strongly increased LEE1 promoter activity (approximately 14-fold compared to EHEC grown in LB and approximately 7-fold compared to DMEM-induced, non-adherent bacteria). Similarly to what we observed with the P_{LEE1}-*gfp* reporter strain, induction of P_{LEE1}-*lacZ* was GrlA-dependent (Figure S2B). LEE1 induction was also observed using P_{LEE1}-*gfp* and P_{LEE1}-*lacZ* transcription reporters in wild type, but not *ΔgrlA* strains, upon bacterial attachment to Caco-2 intestinal epithelial cells, similar to what was observed in Hela cells (approximately 10-fold induction compared to DMEM-induced, non adherent bacteria, Figure S3). The *ΔgrlA* strain showed significantly lower levels of attachment and pedestal formation compared to the wild type strain. However, the overall level of bacterial attachment was lower in Caco-2 cells compared to Hela cells.

Attachment-dependent LEE1 promoter activation is bacteria-driven and is independent of the host response to infection. Stable attachment of EHEC to host cells is a multifactorial process and is the result of a complex interplay between bacterial and host cell signaling. This raises the question if GrlA-dependent LEE1 induction is driven by bacterial signaling alone, or if host-derived signals which form part of the host response to infection are required, too. First, we tested if *de novo* protein synthesis in the host cells was required for attachment-dependent LEE1 induction. Pre-treatment of Hela cells with cycloheximide prior to infection did not change the overall infection phenotype, nor did it alter LEE1 induction levels (Figure 2). Next, we asked whether host cytoskeletal rearrangements leading to pedestal formation were required for LEE1 induction. We analyzed infection phenotype and LEE1 promoter activity in EHEC wild type infected Hela cells after pre-treatment with cytochalasin D, which inhibits actin polymerization and thus pedestal formation. Although cytochalasin D treatment abolished pedestal formation, neither overall bacterial attachment, nor LEE1 activation were affected by the drug-treatment (Figure 2C-G). We conclude that LEE1 promoter activation is likely bacteria-driven as it does not require cues based on de novo protein synthesis or actin rearrangements derived from the host cells as a result of infection.

LEE1 activation results directly from host attachment and is not the result of positive selection for stochastic LEE1 activation through adhesion. Arguably, the selective induction of LEE1 we observe in host-adherent cells could be brought about by at least two different mechanisms: LEE1 induction could be due to host attachment, and thus adhesion would act as a cue for induction. The second scenario is stochastic LEE1 activation in non-adherent cells and then positive selection of bacteria with high LEE activation levels for host attachment, through their enhanced capability to engage with the host cell surface. To distinguish between these two mechanisms, we measured LEE1 induction using a fluorescence plate assay. EHEC wild type strain containing either promoterless *gfp*, inducible P_{LEE1}-*gfp* or constitutively active P_{LEE1}*99T*-*gfp* were incubated in a plate either in the presence or absence of host cells, and total fluorescence per well measured over time. In the presence of host cells, fluorescence of the constitutively active reporter was initially high and slightly increased over the four hour course of the experiment, reflecting bacterial proliferation (Figure 3A). Fluorescence of the promoterless

reporter (background fluorescence) remained low over the same time course. Fluorescence from the inducible LEE1 promoter (P_{LEE1}-*gfp)* was initially low, but increased significantly over the course of the experiment, to reach levels to match those of the constitutive reporter at four hours. The rate of fluorescence increase over time was thus much higher for the P_{LEE1}-*gfp* than the P_{LEE1}*99T-gfp* reporter strain, indicating LEE1 induction rather than an increase due to cell proliferation alone. In the absence of host cells, both rates matched, indicating that LEE1 induction was a result of host attachment rather than selective attachment to host cells due to adhesion-independent stochastic activation (Figure 3B). No significant increase in the fluorescence rate of the P_{LEE1}-*gfp* reporter was observed in a *ΔgrlA* background, even in the presence of host cells (Figure 3C). Since the growth rates of both wild type and mutants strains are similar (Figure S4), this confirms the GrlA-dependence of adhesion-dependent LEE1 induction. We further tested EHEC deletion strains deficient for either Tir (*Δtir*) or Intimin (*Δeae*), two factors involved in stable attachment of EHEC to host cells. Neither of these two mutants showed an increased rate of fluorescence (and thus LEE1 induction) compared to P_{LEE1}*99T-gfp* (Figure 3D, E). Growth rates were unaffected by either *tir* or *eae* deletion (Figure S4). Taken together, these data better align with a scenario in which host-attachment precedes and acts as a cue for LEE1 induction.

Attachment-dependent activation via GrlA underlies positive feedback regulation. EHEC produces several adhesins that facilitate its interaction with host cells, including fimbriae and Tir/Intimin (20). Since both Intimin and its type III-secreted receptor, Tir, are part of the LEE regulon, we investigated if attachment underlies positive feedback regulation. Deletion of either *tir* or *eae*, encoding Tir and Intimin respectively, decreased host-adhesion significantly, both at early (one hour) and later (four hour) time points (Figure 4). The *grlA* deletion mutant showed no significant difference in its initial attachment to host cells. However, after four hours of infection, the number of host-adherent bacteria was significantly decreased (approximately 4-fold) compared to wild type bacteria. This coincides with the time frame for full LEE1 induction (Figure 3A).

The LEE1 promoter is mechanoresponsive and its induction is independent of the mode of attachment. In a bid to identify if a specific host receptor is required for attachment-dependent

198 LEE1 induction, we immobilized EHEC on a range of pure substrates, each mimicking a
199 different type of interaction between bacteria and host cell surface. These included electrostatic
200 interactions between the negatively charged bacterial cell wall and positively charged poly-L-
201 lysine, Tir-Intimin interaction and immobilization using an antibody recognizing the O-antigen
202 moiety of EHEC lipopolysaccharide. Immobilization on all three types of substrates induced
203 LEE1 in a GrlA-dependent manner, albeit to different degrees (Figure 5). In contrast, treatment
204 of bacteria with these adhesion substrates in solution had no significant effect on LEE1 induction
205 (Figure S5). However in each case, exposure of substrate-immobilized bacteria to increasing
206 levels of fluid shear (0.1-10 dynes/cm^2) caused a further increase in LEE1 promoter activity
207 compared to the activity observed under static conditions. Although this behavior was
208 independent of the mechanism of bacteria-substrate interaction, the rate of induction with
209 increasing fluid shear varied depending on the substrate used for immobilization, but saturated at
210 approximately 17000 AFU per cell (corresponding to 7-fold induction compared to static
211 conditions), (Figure 5D, H, L). The number of immobilized bacteria per field did not change
212 significantly with increasing fluid shear, meaning bacteria could withstand the increasing shear
213 force and remained stably attached to the substrate in each case. The level of substrate attachment
214 did not generally alter between wild type and *grlA* deletion mutant, except for bacteria
215 immobilized on Tir peptide, in which case attachment was lower for the *ΔgrlA* strain but also
216 remained stable with increased shear force (Figure S6).

217 To analyze LEE1 induction and phenotypic changes during infection, host-adherent
218 EHEC strains were exposed to increasing levels of fluid shear (Figure 6). Using imaging analysis
219 of *gfp*-reporter strains attached to Hela cells, we observed gradual LEE1 induction in a GrlA-
220 dependent manner under increasing levels of fluid shear (0.1 to 10 dynes/cm^2). The level of LEE1
221 induction increased under fluid shear compared to static conditions, but saturated at
222 approximately 19000 AFU per cell (corresponding to 3.5-fold induction compared to static
223 conditions) and did not further increase under shear flows of up to 10 dynes/cm^2 (Figure 6B).
224 This increase in LEE1 induction in response to fluid shear was partially mirrored by a change in
225 infection phenotype, with more attached bacteria progressing to stable attachment (i.e., pedestal
226 formation) under flow compared to static conditions (Figure 6C, D). Non-adherent bacteria
227 exposed to flow conditions did not show increased levels of LEE1 induction (Figure 6E).

Only free, but not GrlR-bound GrlA is mechanoresponsive. It is well documented that GrlR acts as a repressor of GrlA-mediated LEE1 promoter induction, and thus LEE activation, by sequestering a portion of the cell's GrlA in a $(GrlR)_2$-GrlA complex (21). We therefore tested whether both free and GrlR-bound pools of GrlA are mechanoresponsive. If host attachment acts on the GrlRA complex to relieve GrlR-mediated repression, deletion of *grlR* should mimic the effect of host attachment. We thus compared LEE1 induction in EHEC wild type and Δ*grlR* strains containing P_{LEE1}-*lacZ* transcriptional fusions. Deletion of *grlR* enhanced LEE1 induction by approximately 2-fold, but did not mimic the strong induction seen in host-adherent bacteria (Figure S7). This suggests that attachment-mediated activation of GrlA is not achieved merely by relieving GrlR-mediated suppression of GrlA, and other, GrlR-independent modes of regulating GrlA activity exist.

We also analyzed LEE1 promoter activity and infection phenotype in EHEC wild type cells over-expressing either GrlR, both GrlR and GrlA, or GrlA alone. Cells infected with EHEC expressing additional GrlR showed a very similar phenotype to cells infected with the Δ*grlA* strain – P_{LEE1}-*gfp* activity, number of attached bacteria and pedestal formation were significantly decreased compared to cells infected with EHEC wild type bacteria (Figure 7A). GrlA overexpression, on the other hand, led to a hyperinfective phenotype, with an approximately two-fold increase in both the number of attached bacteria and pedestals formed (Figure 7C), but this phenotype was not recapitulated with the GrlRA overexpressing strain (Figure 7B), which behaved similar to the EHEC wild type strain. These results were recapitulated using P_{LEE1}-*lacZ* reporter strains overexpressing either GrlR, GrlRA, or GrlA (Figure 7H). LEE1 induction was slightly enhanced in both the GrlRA and GrlA overexpressing wild type cells harvested from the supernatant during infection, or from cells grown in planktonic cultures. This slight enhancement in LEE1 induction was exaggerated by host-attachment, where GrlA overexpression caused an approximately 13-fold induction of LEE1 over wild type cells (which, themselves, show a 14-fold induction compared to planktonic cells). These data confirm that only free GrlA is mechanoresponsive and can induce LEE1, while GrlRA complex remains unaffected by this stimulus. Our data also suggests that the cellular pool of free GrlA is not, in itself, competent to fully induce LEE1, but becomes activated as a result of host attachment via an as yet unidentified mechanism.

DISCUSSION

Human disease caused by EHEC infection is usually the result of food-borne transmission. Thus, bacteria exit the ruminant gastrointestinal tract and persist on contaminated food matter, before being taken up into a human host, where they colonize and cause diarrheal disease. Following human uptake, bacteria are exposed to a range of host-specific cues, including a shift in temperature, passage through the acidic stomach environment, neutralization through bicarbonate exposure and finally, the intestinal environment. It has always been assumed that sequential exposure to these host-specific triggers is sufficient to induce virulence exclusively within the human host niche, the intestine. Previous studies have indeed demonstrated induction of Ler and thus LEE, in response to environmental stimuli. For example, GrlA is expressed in response to bicarbonate released by the pancreas and this partially induces LEE and thus virulence (22, 23). Here, we show that while the levels of GrlA have a subtle effect on Ler activation, full virulence induction is only achieved through host attachment. This departs from our previous understanding of GrlA-based regulation, which was thought to require GrlR for inhibition and release of GrlA from the GrlR complex to achieve activation. In contrast to this, our data give strong evidence supporting the hypothesis that full induction by GrlA relies on mechanically stimulated activation of free GrlA, while the same cue does not activate GrlR-bound GrlA. How exactly GrlA becomes competent to bind to or activate the LEE1 promoter is clearly more coplex than a transition from GrlR-bound to unbound states. It could be due to a change in subcellular localization, post-translational modification, or additional binding partners, and these possibilities will be addressed in future work. This mechanism of virulence induction underlies positive feedback regulation, since the LEE includes both Tir and Intimin, factors required for intimate host attachment. While EHEC adhesion is mediated by multiple components and thus LEE induction does not strictly require Tir/Intimin, their presence reinforces existing bacterial attachment and thus optimizes mechanotransduction.

Taken together our data suggests that, while exposure to early host environmental triggers may cause basal activation of the LEE and thus poise the system to respond, full activation of virulence requires two components of mechanosensation: First, direct contact with and attachment to the host cell surface, which contributed to an approximately 7-fold induction over host exposed but non-attached bacteria. Second, enhancement of the thus generated force in

response to fluid shear levels comparable to those in the intestinal lumen, which leads to a further 3-4 fold activation of LEE1 in bacteria experiencing fluid shear, compared to static conditions. Levels of fluid shear in the intestinal tract vary, depending on the exact physical location. According to hydrodynamic calculations, shear forces can approach 5 dynes/cm^2 on the exposed brush border surface, and decrease to 2-3 dynes/cm^2 between microvilli, depending on the flow rate (24). This highlights the physiological relevance of the LEE1 induction observed in our experiments, which reaches its maximum around 1 dyne/cm^2. The basic physical sensation of mechanical forces thus acts to integrate a variety of host-specific, chemical signals and ensures the complex arsenal of virulence factors is only fully expressed once the pathogen has reached its dedicated niche. While such chemical stimuli may vary between different environments and even different host organisms, these physical parameters are a conserved signal indicating the presence of a host surface.

Further work will be needed to understand what bacterial envelope components are involved in transduction of the mechanical signal sensed at the outer membrane in response to attachment, to GrlA, the cytoplasmic regulator of virulence genes. The plate-based fluorescence assay used here to measure promoter activation in response to attachment (Figure 3) can be easily adopted to conduct high-throughput screens to identify further bacterial components involved in signal perception and transduction across the bacterial cell envelope. The EHEC surface contains multiple mechanoresponsive elements and factors which could have a putative role in signaling attachment, including flagella (during the early stages of attachment), fimbrial adhesins or, as recently reported, PilY (16, 25-27). Recently, Siryaporn et al described mechanosensing as the inducing signal for virulence in *Pseudomonas aeruginosa*, and implicated PilY as the outer membrane component of the signal transduction pathway, although further components of the transduction mechanism remained elusive (16). In comparison to attaching/effacing pathogens such as EHEC, *P. aeruginosa* colonizes different niches within the host and comprises a different arsenal of virulence mechanisms. Yet, surface attachment equally acts as a general and evolutionary conserved signal for the presence of a host cell. This opens up the exciting perspective that mechanoperception is an evolutionary robust and widely employed principle utilized by microbial pathogens to integrate a large and divergent set of specific environmental cues.

MATERIALS AND METHODS

The wild type strain used in this study was an EHEC O157:H7 Sakai shiga-toxin negative derivative strain (Sakai 813), a derivative of RIMD 0509952 (28). The gene-doctoring procedure was used to introduce gene deletions in this background, as previously described (29). All described strains and plasmids are listed in Table S1. Details of growth conditions, infection experiments under static and flow conditions, surface coating, imaging and measurements of transcriptional activity are described in the Supplementary Information, SI Materials and Methods.

ACKNOWLEDGMENTS

We thank S. Sasakawa for the kind gift of EHEC strain Sakai 813. We thank Dave Lee, Jack Bryant and Laura Sellars for technical assistance with construction of transcriptional fusions and EHEC deletion strains. We thank Mark Webber for providing equipment access and technical assistance with flow cell experiments. We thank members of the Krachler and Busby labs for critical reading and comments on the manuscript. This work was supported by grants from the BBSRC (to A.M.K. and S.J.W.B.), the Leverhulme Trust (to S.J.W.B), a King Abdulaziz University Scholarship (to G.A.) and a Commonwealth Academic Fellowship (to M.S.I.).

REFERENCES

1. Nataro JP & Kaper JB (1998) Diarrheagenic Escherichia coli. *Clinical microbiology reviews* 11(1):142-201.
2. McDaniel TK & Kaper JB (1997) A cloned pathogenicity island from enteropathogenic Escherichia coli confers the attaching and effacing phenotype on E. coli K-12. *Molecular microbiology* 23(2):399-407.
3. Jerse AE, Yu J, Tall BD, & Kaper JB (1990) A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Sciences of the United States of America* 87(20):7839-7843.
4. McDaniel TK, Jarvis KG, Donnenberg MS, & Kaper JB (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 92(5):1664-1668.
5. Elliott SJ, *et al.* (2000) The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic Escherichia coli. *Infection and immunity* 68(11):6115-6126.
6. Elliott SJ, *et al.* (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic Escherichia coli E2348/69. *Molecular microbiology* 28(1):1-4.
7. Deng W, *et al.* (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proceedings of the National Academy of Sciences of the United States of America* 101(10):3597-3602.
8. Bustamante VH, Santana FJ, Calva E, & Puente JL (2001) Transcriptional regulation of type III secretion genes in enteropathogenic Escherichia coli: Ler antagonizes H-NS-dependent repression. *Molecular microbiology* 39(3):664-678.
9. Sperandio V, Torres AG, Jarvis B, Nataro JP, & Kaper JB (2003) Bacteria-host communication: the language of hormones. *Proceedings of the National Academy of Sciences of the United States of America* 100(15):8951-8956.
10. Pacheco AR, *et al.* (2012) Fucose sensing regulates bacterial intestinal colonization. *Nature* 492(7427):113-117.
11. Yoh M, Bi Z, Matsuyama J, Nagayama K, & Honda T (2003) Effect of environmental conditions on proteins secreted by enterohemorrhagic Escherichia coli O26:H11. *Microbiology and immunology* 47(1):1-6.
12. Branchu P, *et al.* (2014) NsrR, GadE, and GadX interplay in repressing expression of the Escherichia coli O157:H7 LEE pathogenicity island in response to nitric oxide. *PLoS pathogens* 10(1):e1003874.
13. Barba J, *et al.* (2005) A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. *Journal of bacteriology* 187(23):7918-7930.
14. Iyoda S, *et al.* (2006) The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohemorrhagic Escherichia coli. *Journal of bacteriology* 188(16):5682-5692.
15. Russell RM, Sharp FC, Rasko DA, & Sperandio V (2007) QseA and GrlR/GrlA regulation of the locus of enterocyte effacement genes in enterohemorrhagic Escherichia coli. *Journal of bacteriology* 189(14):5387-5392.
16. Siryaporn A, Kuchma SL, O'Toole GA, & Gitai Z (2014) Surface attachment induces Pseudomonas aeruginosa virulence. *Proceedings of the National Academy of Sciences of the United States of America* 111(47):16860-16865.
17. 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. *Molecular microbiology* 79(2):468-483.

18. Tobe T, *et al.* (2005) Dual regulatory pathways integrating the RcsC-RcsD-RcsB signalling system control enterohaemorrhagic Escherichia coli pathogenicity. *Molecular microbiology* 58(1):320-333.
19. Knutton S, Baldwin T, Williams PH, & McNeish AS (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli. *Infection and immunity* 57(4):1290-1298.
20. Farfan MJ, Cantero L, Vidal R, Botkin DJ, & Torres AG (2011) Long polar fimbriae of enterohemorrhagic Escherichia coli O157:H7 bind to extracellular matrix proteins. *Infection and immunity* 79(9):3744-3750.
21. Padavannil A, *et al.* (2013) Structure of GrlR-GrlA complex that prevents GrlA activation of virulence genes. *Nature communications* 4:2546.
22. Abe H, Tatsuno I, Tobe T, Okutani A, & Sasakawa C (2002) Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic Escherichia coli O157:H7. *Infection and immunity* 70(7):3500-3509.
23. Tauschek M, *et al.* (2010) Transcriptional analysis of the grlRA virulence operon from Citrobacter rodentium. *Journal of bacteriology* 192(14):3722-3734.
24. Guo P, Weinstein AM, & Weinbaum S (2000) A hydrodynamic mechanosensory hypothesis for brush border microvilli. *American journal of physiology. Renal physiology* 279(4):F698-712.
25. Cairns LS, Marlow VL, Bissett E, Ostrowski A, & Stanley-Wall NR (2013) A mechanical signal transmitted by the flagellum controls signalling in Bacillus subtilis. *Molecular microbiology* 90(1):6-21.
26. Thomas WE, Trintchina E, Forero M, Vogel V, & Sokurenko EV (2002) Bacterial adhesion to target cells enhanced by shear force. *Cell* 109(7):913-923.
27. Tchesnokova V, *et al.* (2010) Shear-enhanced binding of intestinal colonization factor antigen I of enterotoxigenic Escherichia coli. *Molecular microbiology* 76(2):489-502.
28. Makino K, *et al.* (1998) Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic Escherichia coli O157:H7 derived from Sakai outbreak. *DNA research : an international journal for rapid publication of reports on genes and genomes* 5(1):1-9.
29. Lee DJ, *et al.* (2009) Gene doctoring: a method for recombineering in laboratory and pathogenic Escherichia coli strains. *BMC microbiology* 9:252.

FIGURES & LEGENDS

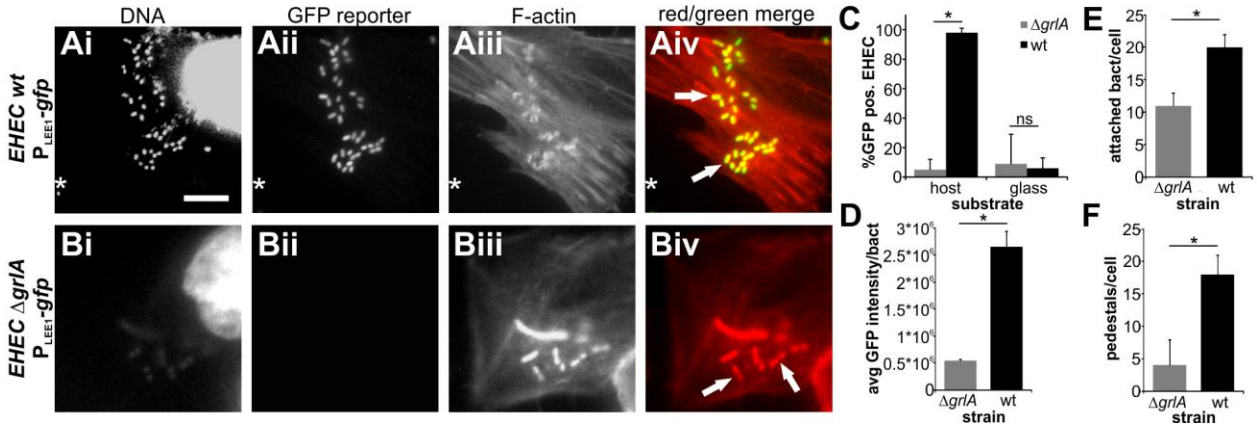


Figure 1. Attachment to host cells triggers LEE1 promoter activation in a GrlA-dependent manner. EHEC wild type (A) or EHEC Δ*grlA* (B) harboring a P_{LEE1}-*gfp* transcriptional fusion as reporter were used to infect Hela cells (MOI 10, 4 hrs). Samples were fixed and DNA (Hoechst), reporter activation (GFP) and F-actin (rhodamine-phalloidin) were visualized by fluorescence microscopy. Several actin pedestals caused by EHEC attachment are marked by arrows. Example of an EHEC bacterium adsorbed to the glass slide, rather than attached to host cells, is marked by an asterisk. The scale bar represents 10 µm. % GFP positive bacteria (C), average GFP intensity per bacterium (for GFP positive cells), (D), number of attached bacteria/host cell (E) and number of pedestals/host cell (F) 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 ($p \geq 0.05$).

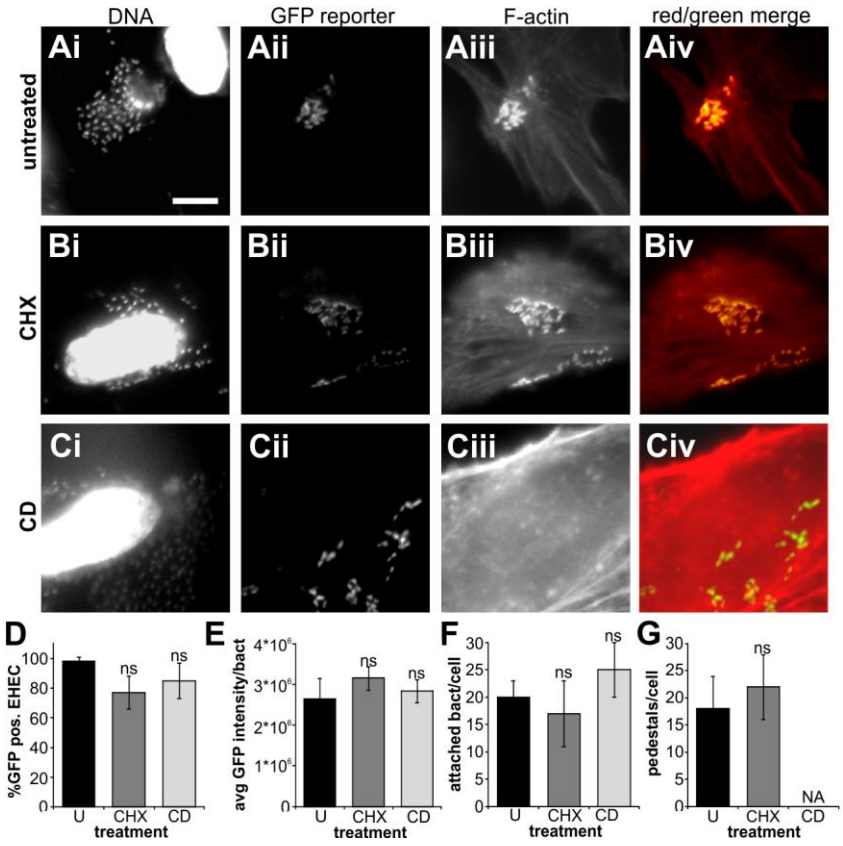


Figure 2. Induction of LEE1 is bacteria-driven and a host response to infection is not required for signal transmission to GrlA. Hela cells were infected with EHEC harboring a P_{LEE1}-*gfp* transcriptional fusion (MOI 10, 4 hours) following pre-treatment with either DMSO as control (A), 10 µg/ml cycloheximide (B) or 1 µg/ml cytochalasin D (C) for 1 hour. Samples were fixed and DNA (Hoechst), reporter activation (GFP) and F-actin (rhodamine-phalloidin) were visualized by fluorescence microscopy. The scale bar represents 10 µm. % GFP positive bacteria (D), average GFP intensity per bacterium (for GFP positive cells), (E), number of attached bacteria/host cell (F) and number of pedestals/host cell (G) were determined for untreated (U), cycloheximide-treated (CHX) and cytochalasin D-treated (CD) cells. 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 (no pedestals formed in CD-treated cells).

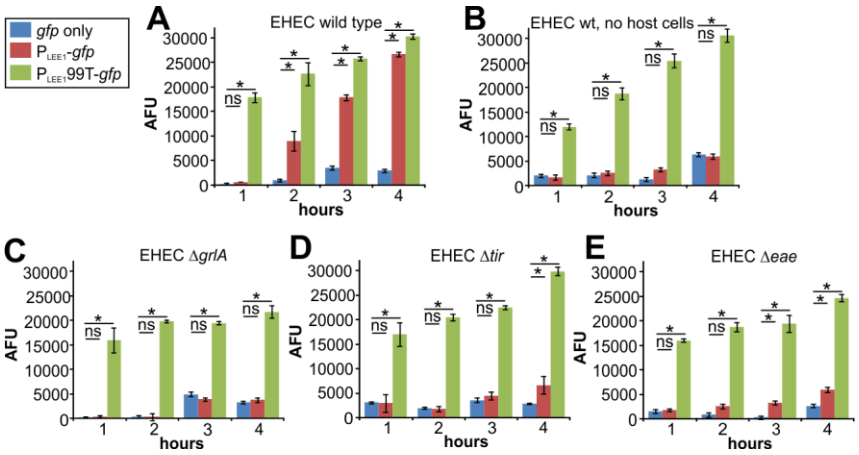


Figure 3. Population level analysis of LEE1 induction rates in EHEC wild type and mutant strains. Fluorescence intensity (AFU) was measured as a read-out for promoter activation using promoterless *gfp* (blue), P_{LEE1}-*gfp* (red) or P_{LEE1}*99T-gfp* (green) reporter constructs in EHEC wild type cells grown in the presence (A) or absence (B) of host cells. Fluorescence was also measured in EHEC Δ*grlA* (C), Δ*tir* (D) and Δ*eae* (E) strains incubated in the presence of Hela cells for 1, 2, 3 or 4 hours. Data are representative of three independent experiments done in triplicate. Asterisks denote significant differences between samples based on student's t-test ($p < 0.05$). ns; not significant ($p \geq 0.05$).

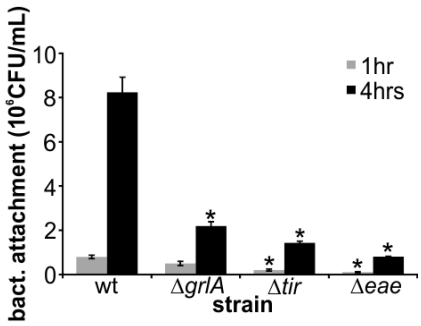


Figure 4. Bacterial attachment over time in EHEC wild type and deletion strains. Hela cells were infected with EHEC wild type or deletion strains (MOI of 10) and bacterial attachment to host cells was determined after 1 (grey bars) or 4 hours (black bars) of infection by dilution plating. Data are representative of three independent experiments done in triplicate. The asterisk denotes significant differences between wild type and deletion strains at the respective time point, based on student's t-test ($p < 0.05$). ns; not significant ($p \geq 0.05$).

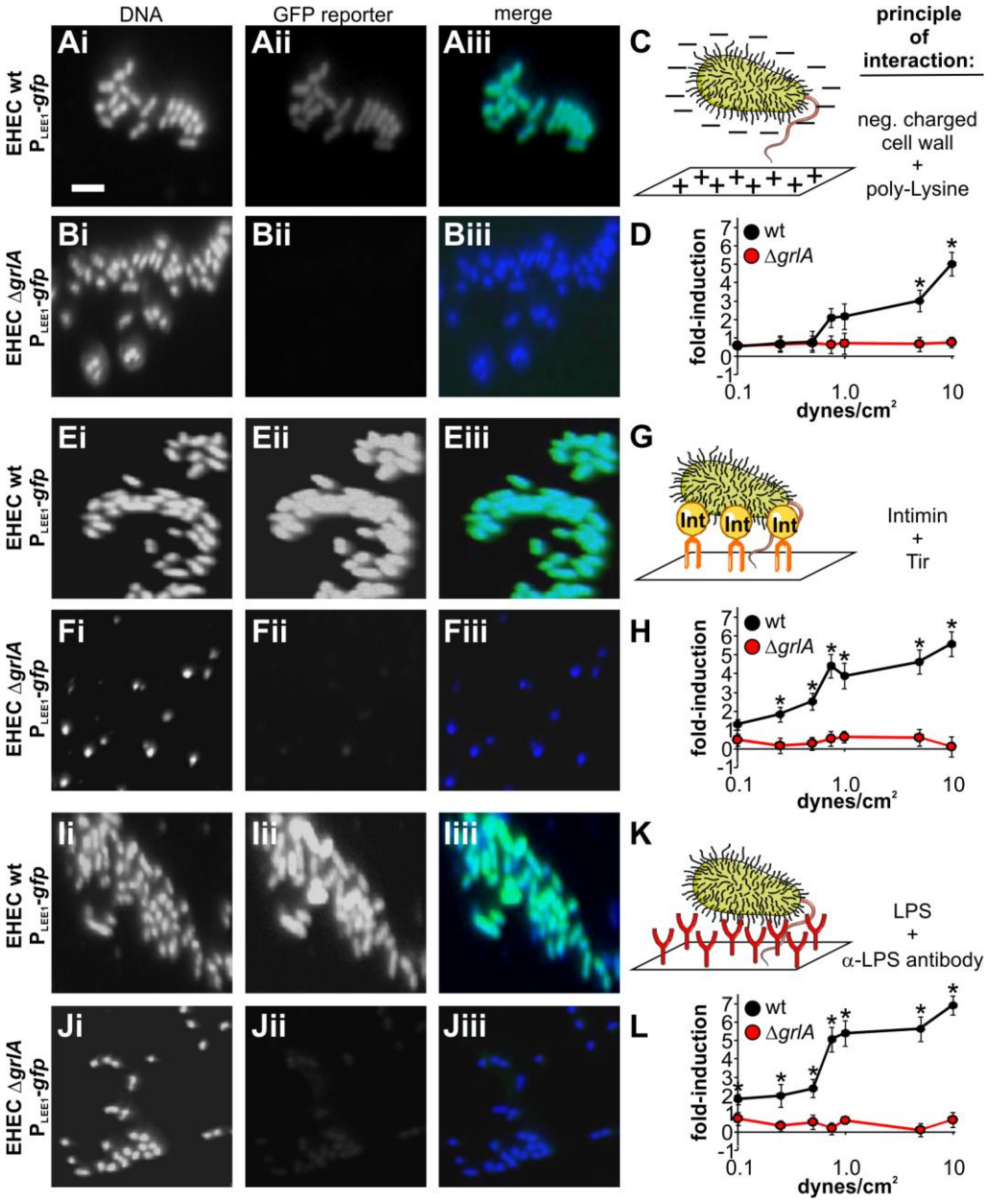


Figure 5. LEE1 induction is independent of the mode of attachment but the shape of the force response curve is substrate-dependent. EHEC wild type (A, E, I) or Δ*grlA* (B, F, J) strains containing a P_{LEE1}-*gfp* reporter were introduced into substrate-coated flow cells and incubated for 1 hour under static conditions, followed by 3 hours of flow to give a defined fluid shear force ranging from 0-10 dynes/cm^2. Substrates included poly-L-lysine (A-D), Tir-peptide (E-H) and α-LPS antibody (I-L) and were chosen to represent different modes of bacterial attachment. Images are representative of bacteria incubated under static conditions (0 dynes/cm^2). Scale bar, 5 μm. Following the experiment, average fluorescence intensity (AFU) per bacterium

was determined from image analysis and values blotted as fold-change compared to wt EHEC on poly-K under static conditions (D, H, L). Data are representative of three independent experiments (> 100 cells each). The asterisk denotes significant differences between samples based on student's t-test ($p < 0.05$).

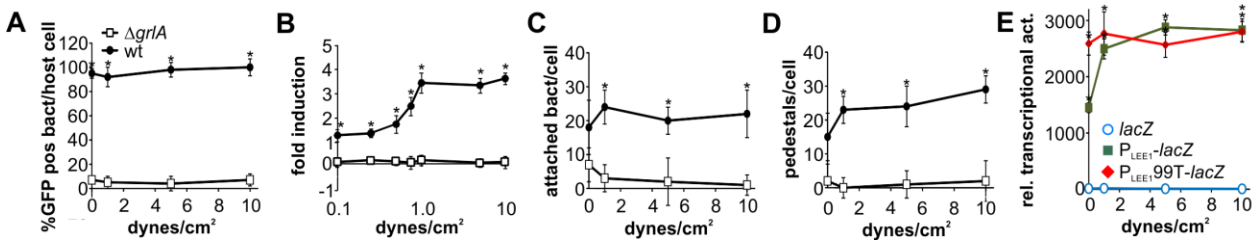


Figure 6. Fluid shear exacerbates LEE1 activation in host-attached bacteria. EHEC wild type (black circles) or Δ*grlA* strains (white squares) containing a P_{LEE1}-*gfp* reporter were used to infect Hela cells grown in glass flow cells and incubated for 1 hour under static conditions, followed by 3 hours of flow to give a defined fluid shear force ranging from 0-10 dynes/cm^2. Following the experiment, % GFP positive bacteria/cell (A), fold-change in average GFP intensity per bacterium compared to static conditions (B), attached bacteria/cell (C) and pedestals/cell (D) were determined from image analysis. Data are representative of three independent experiments (> 100 Hela cells each). Hela cells grown in glass flow cells were also infected with EHEC wild type strain containing either promoter-less *lacZ* (blue), P_{LEE1}-*lacZ* (green) or P_{LEE1}*99T-lacZ* (red) reporters, as described above. Following the experiment, cells were detached from the flow cells using Triton-X100, and samples used to determine relative transcriptional activities (E). Data are representative of three independent experiments performed in triplicate. The asterisk denotes significant differences between samples based on student's t-test ($p < 0.05$).

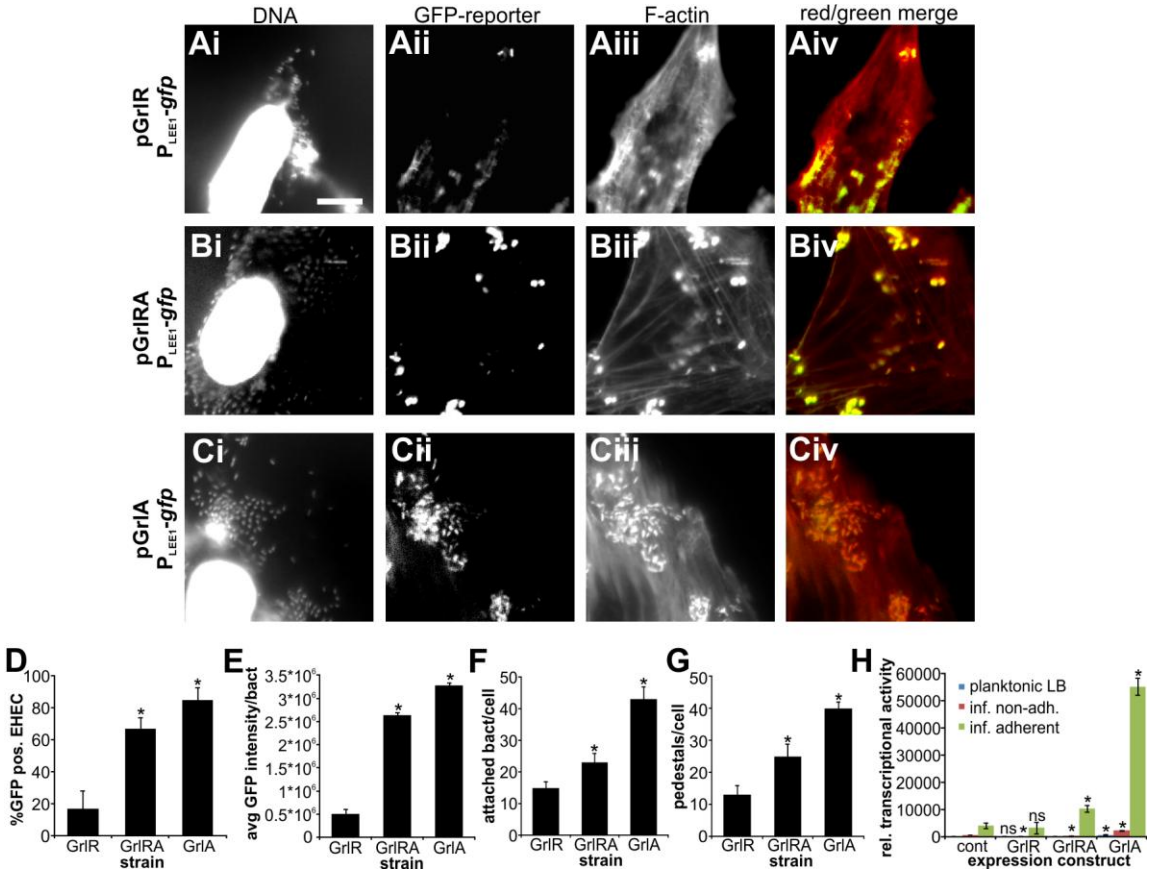


Figure 7. Only free, but not GrlR bound GrlA is competent for attachment-mediated LEE1 induction and attachment does not relieve GrlR-mediated repression of GrlA. EHEC wild type strain harboring a P_{LEE1}-*gfp* transcriptional fusion as reporter and either GrlR (A), GrlRA (B) or GrlA (C) expression vectors were used to infect Hela cells (MOI 10, 4 hrs). Samples were fixed and DNA (Hoechst), reporter activation (GFP) and F-actin (rhodamine-phalloidin) were visualized by fluorescence microscopy. The scale bar represents 10 μm. % GFP positive bacteria (D), average GFP intensity per bacterium (for GFP positive cells), (E), number of attached bacteria/host cell (F) and number of pedestals/host cell (G) were determined from these experiments. Data are representative of three independent experiments (> 100 Hela cells each). Hela cells were also infected (MOI 10, 4 hours) with EHEC wild type strain harboring a P_{LEE1}-*lacZ* transcriptional fusion as reporter and either empty vector (cont), GrlR, GrlRA or GrlA expression constructs (H). Non-adherent 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 results were normalized to CFU/ml and are shown as relative transcriptional activity. Rel. transcriptional activity was also

505 determined for bacteria grown in planktonic LB cultures (blue). The asterisk denotes significant
506 differences between bacteria harboring empty vector and expression constructs, based on
507 student's t-test ($p < 0.05$, n=3). ns; not significant ($p \geq 0.05$).

508

Host attachment and fluid shear are integrated into a mechanical signal regulating virulence in *Escherichia coli* O157:H7

Ghadah Alsharif[1], Sadia Ahmad[1], Md. Shahidul Islam[2], Riddhi Shah[1], Stephen J W Busby[1], Anne Marie Krachler[1,*]

[1]Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, B15 2TT Birmingham, UK

[2]Department of Biotechnology, Bangladesh Agricultural University, BAU Main Road, Mymensingh 2202, Bangladesh

[*]Correspondence to: a.krachler@bham.ac.uk

Classification: Biological Sciences; Microbiology

Keywords: enterohemorrhagic *Escherichia coli*, locus of enterocyte effacement, attaching/effacing pathogens, gastrointestinal infection, mechanosensing, host-pathogen interactions

SUPPLEMENTARY INFORMATION

Supplemental Table S1

Supplemental Figures FigS1-S7

Supplemental Materials and Methods

Table S1. Strains and plasmids used in this study.

Strain or plasmid	Description	Reference
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*	(17)
EHEC Δ*grlR*	EHEC O157:H7 Sakai 813 *Δstx ΔgrlR*	(17)
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	(30)
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	(17)
pRW224/LEE10-568 (P_{LEE1}-*lacZ*)	A derivative of pRW224 carrying an *Eco*RI-*Hind*III LEE1 promoter (position -568 to position -19 relative to the Ler translation start site) as a transcriptional fusion to *lac*Z	(17)

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*	(17)
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 *Eco*RI- *Hin*dIII 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 *Eco*RI-*Hin*dIII 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.	(31)
pSI01 (pGrlRA)	A derivative of pACYC184 carrying the *grlRA* operon including its promoter region cloned into *Hin*dIII and *Sal*I sites	(17)
pSI02 (pGrlA)	A derivative of pSI01 carrying a *grlR* deletion	(17)
pSI03 (pGrlR)	A derivative of pSI01 carrying a *grlA* deletion	(17)

Supplemental Figure Legends

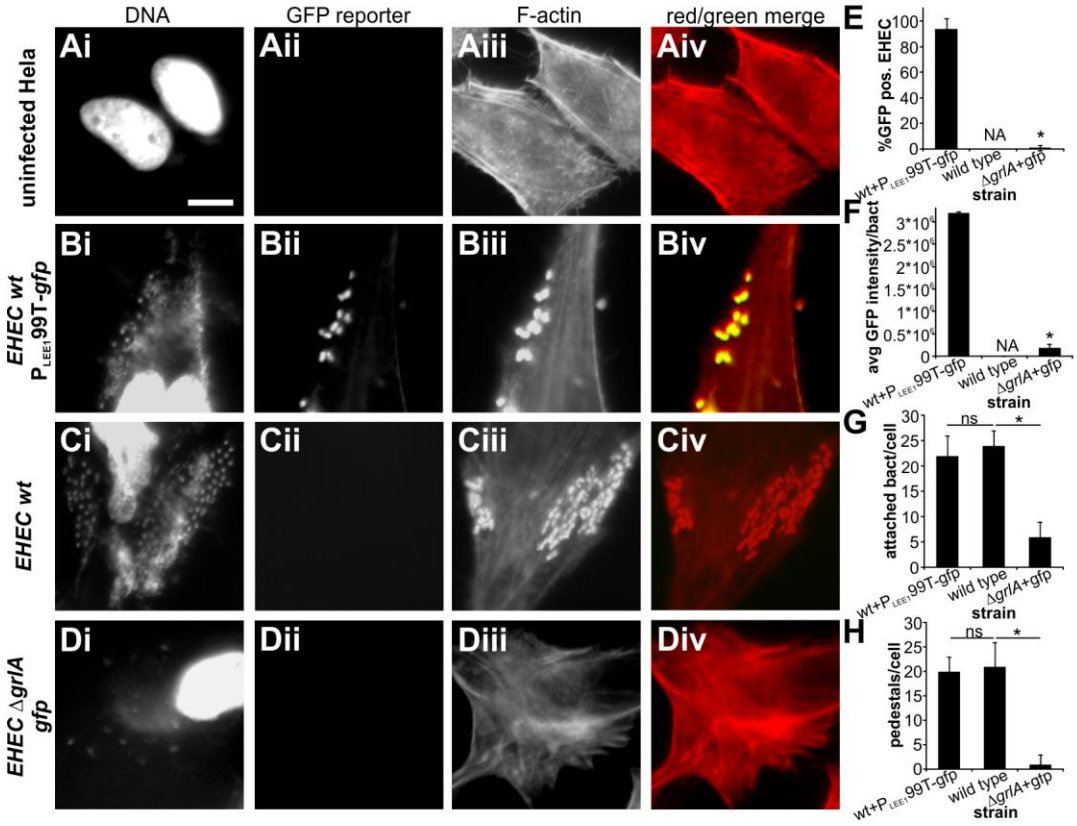
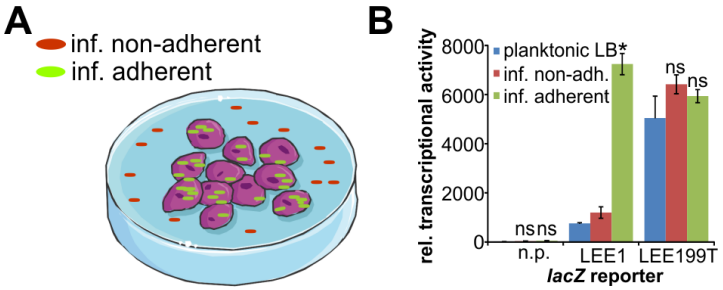


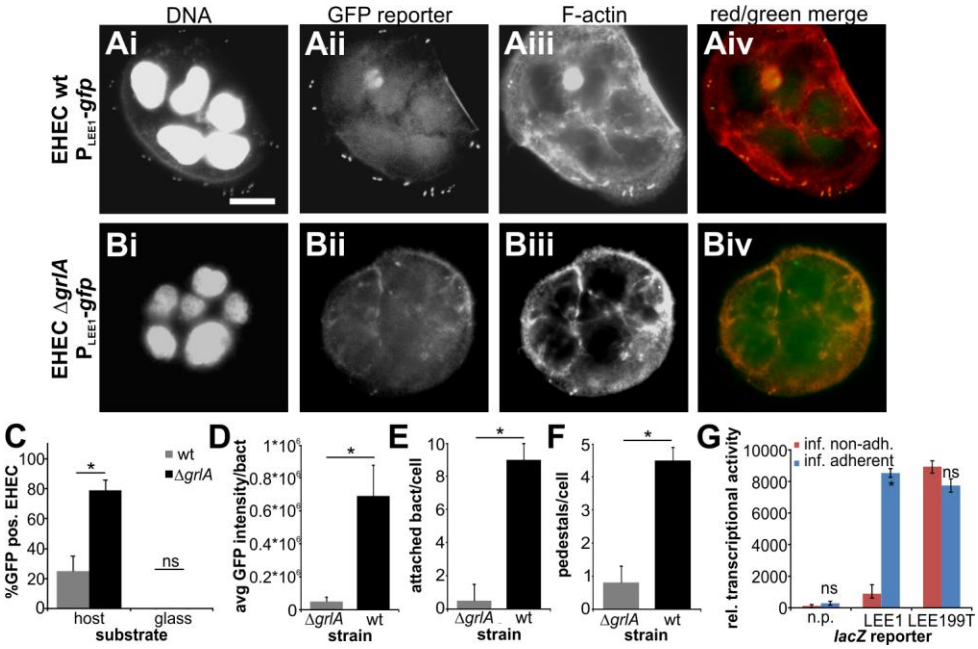
Figure S1. Phenotype and LEE1 promoter induction in EHEC reporter strains infecting Hela cells. Hela cells were either left uninfected (A) or infected with EHEC wild type containing P_{LEE1}99T-*gfp* (B), EHEC wild type (C) or EHEC Δ*grlA* harboring a promoter-less *gfp* reporter (D), at an MOI 10 for 4 hrs. Samples were fixed and DNA (Hoechst), reporter activation (GFP) and F-actin (rhodamine-phalloidin) were visualized by fluorescence microscopy. The scale bar represents 10 μm. % GFP positive bacteria (E), average GFP intensity per bacterium (for GFP positive cells), (F), number of attached bacteria/host cell (G) and number of pedestals/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).



559

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

572



573

574 **Figure S3. Phenotype and LEE1 promoter induction in EHEC reporter strains infecting**
575 **Caco-2 cells.** EHEC wild type (A) or EHEC Δ*grlA* (B) harboring a P_{LEE1}-*gfp* transcriptional
576 fusion as reporter were used to infect Caco-2 cells (MOI 10, 4 hrs). Samples were fixed and DNA
577 (Hoechst), reporter activation (GFP) and F-actin (rhodamine-phalloidin) were visualized by
578 fluorescence microscopy. The scale bar represents 20 μm. % GFP positive bacteria (C), average

GFP intensity per bacterium (for GFP positive cells), (D), number of attached bacteria/host cell (E) and number of pedestals/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 either 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 hours), non-adherent (red) and host-adherent (blue) bacteria separated, β-galactosidase activity determined in each of these fractions and expressed as a function of bacterial counts to give relative transcriptional activities. The asterisk denotes significant differences between non-adherent and adherent fractions based on student's t-test ($p < 0.05$, n=3). ns; not significant ($p \geq 0.05$).

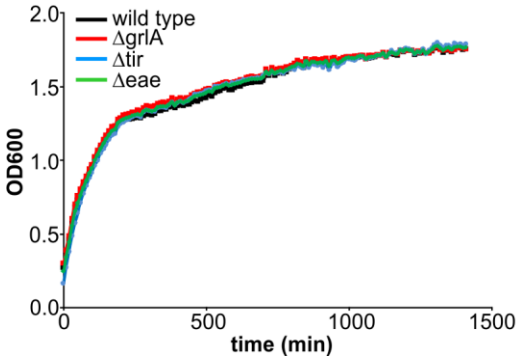


Figure S4. Growth of EHEC wild type and deletion mutants. EHEC wild type or deletion strains were grown overnight in LB broth and diluted into DMEM to give an initial OD_{600} of 0.25. Strains were then grown in a 96-well plate at 37 °C under intermittent shaking and OD_{600} measured every 10 minutes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

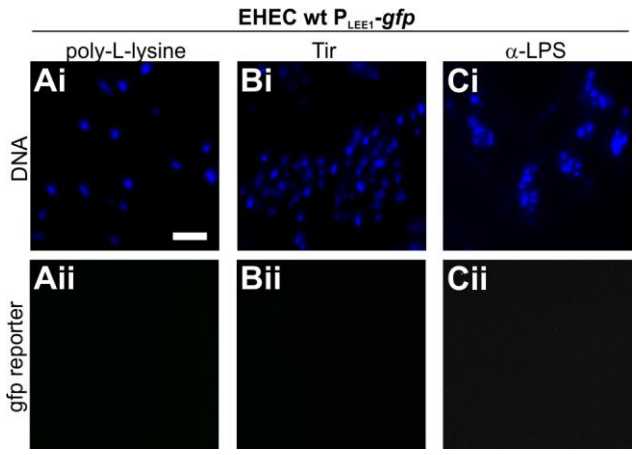


Figure S5. Bacterial attachment to soluble substrates does not cause LEE1 induction. EHEC wild type strain containing a P_{LEE1}-*gfp* reporter was incubated with soluble substrates – either poly-L-lysine (A), Tir-peptide (B) or α-LPS antibody (C) under static conditions for 4 hours prior

to imaging bacteria by DNA staining (Hoechst, top row) and LEE1 activity by GFP fluorescence (bottom row). Scale bar, 5 μm.

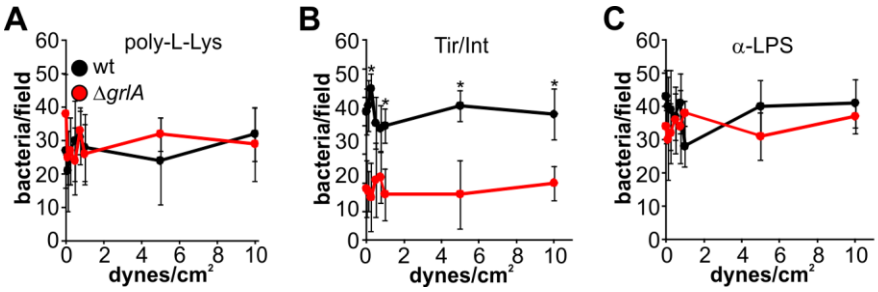


Figure S6. The number of substrate-attached bacteria is independent of fluid shear force. The total number of attached bacteria per field was enumerated for both EHEC wild type (black) and Δ*grlA* (red) strains and for channels coated with either poly-L-lysine (A), Tir-peptide (B) or α-LPS antibody (C). In each case, the total number of bacteria remained constant with increasing fluid shear force between 0-10 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 ($p < 0.05$).

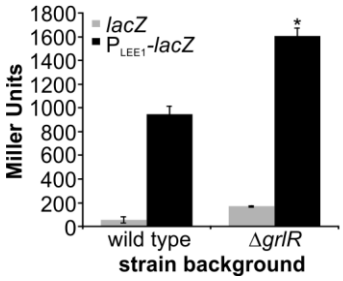


Figure S7. Effect of *grlR* deletion in EHEC on LEE1 induction. LEE1 promoter activity was monitored using either promoterless *lacZ* (grey) or P_{LEE1}-*lacZ* (black) transcriptional fusion constructs in EHEC wild type or Δ*grlR* cells grown in DMEM to an OD_{600} of ~0.5 at 37 °C. Data are representative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

Supplemental Materials and Methods

Strains, Cell lines and Growth Conditions. Bacteria were maintained on MacConkey agar and unless otherwise stated in the figure legends, sub-cultured 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 ºC and under 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 4500 mg/L glucose, 0.5 mM L-glutamine, 100 units/ml penicillin and 20 μg/ml streptomycin.

Infection of host cells under static and flow conditions. Tissue culture cells were washed with PBS (phosphate-buffered saline) prior to the addition of bacteria in tissue culture medium without antibiotics. Bacteria were added to give a multiplicity of infection (MOI) of 10 prior to incubation at 37 ºC for 30 minutes to four hours, depending on the experiment (see figure legends for details). For enumeration of bacteria, samples were removed at time points as indicated and were serially diluted, plated on LB agar plates, incubated at 37 ºC for sixteen hours and colony forming units determined. For enumeration of host-adherent bacteria, host cells were washed three times with PBS and lysed with PBS containing 1% Triton X-100 prior to dilution plating. For flow experiments, host cells were cultured in flow cells one day prior to infection. To infect, EHEC were introduced onto the host cell layer, the flow discontinued and flow cells left at 37 ºC for 1 hour under static conditions. Fresh DMEM was then flowed across the cell layers at variable flow rates, to result in shear forces from 0-10 dynes/cm^2. Flow cells were then either perfused with 3.2% paraformaldehyde to fix samples prior to imaging, or with PBS+1% Triton X-100 to harvest samples for plating and β-galactosidase assays, as described below.

Imaging of EHEC infections. For microscopy, samples were fixed with 3.2% formaldehyde, permeabilized with 0.1% Triton X-100 and stained for 10 minutes 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 X5.

Surface coating with pure substrates for bacterial adhesion. Cover slips and flow cell surfaces were coated with either 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 (0.2mg/ml aqueous solution) for 1 hour at 22 °C. Solution was aspirated and surface left to dry for 1 hour at 37 °C. Surface was rinsed with PBS prior to bacterial attachment. For coating with Tir peptide, His-Tir-M was prepared as described previously (32), 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 rinsed with PBS prior to 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 approximately 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 one, two, three or four hours. Each sample was measured in triplicate wells and at least three independent experiments were performed.

Supplemental References

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. *Molecular microbiology* 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 microbiology letters* 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. *Journal of bacteriology* 134(3):1141-1156.

1. Nataro JP & Kaper JB (1998) Diarrheagenic Escherichia coli. *Clinical microbiology reviews* 11(1):142-201.
2. McDaniel TK & Kaper JB (1997) A cloned pathogenicity island from enteropathogenic Escherichia coli confers the attaching and effacing phenotype on E. coli K-12. *Molecular microbiology* 23(2):399-407.
3. Jerse AE, Yu J, Tall BD, & Kaper JB (1990) A genetic locus of enteropathogenic Escherichia coli necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Sciences of the United States of America* 87(20):7839-7843.
4. McDaniel TK, Jarvis KG, Donnenberg MS, & Kaper JB (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Sciences of the United States of America* 92(5):1664-1668.
5. Elliott SJ*, et al.* (2000) The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic Escherichia coli. *Infection and immunity* 68(11):6115-6126.
6. Elliott SJ*, et al.* (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic Escherichia coli E2348/69. *Molecular microbiology* 28(1):1-4.
7. Deng W*, et al.* (2004) Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proceedings of the National Academy of Sciences of the United States of America* 101(10):3597-3602.
8. Bustamante VH, Santana FJ, Calva E, & Puente JL (2001) Transcriptional regulation of type III secretion genes in enteropathogenic Escherichia coli: Ler antagonizes H-NS-dependent repression. *Molecular microbiology* 39(3):664-678.
9. Sperandio V, Torres AG, Jarvis B, Nataro JP, & Kaper JB (2003) Bacteria-host communication: the language of hormones. *Proceedings of the National Academy of Sciences of the United States of America* 100(15):8951-8956.
10. Pacheco AR*, et al.* (2012) Fucose sensing regulates bacterial intestinal colonization. *Nature* 492(7427):113-117.
11. Yoh M, Bi Z, Matsuyama J, Nagayama K, & Honda T (2003) Effect of environmental conditions on proteins secreted by enterohemorrhagic Escherichia coli O26:H11. *Microbiology and immunology* 47(1):1-6.
12. Branchu P*, et al.* (2014) NsrR, GadE, and GadX interplay in repressing expression of the Escherichia coli O157:H7 LEE pathogenicity island in response to nitric oxide. *PLoS pathogens* 10(1):e1003874.
13. Barba J*, et al.* (2005) A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. *Journal of bacteriology* 187(23):7918-7930.

14. Iyoda S*, et al.* (2006) The GrlR-GrlA regulatory system coordinately controls the expression of flagellar and LEE-encoded type III protein secretion systems in enterohemorrhagic Escherichia coli. *Journal of bacteriology* 188(16):5682-5692.
15. Russell RM, Sharp FC, Rasko DA, & Sperandio V (2007) QseA and GrlR/GrlA regulation of the locus of enterocyte effacement genes in enterohemorrhagic Escherichia coli. *Journal of bacteriology* 189(14):5387-5392.
16. Siryaporn A, Kuchma SL, O'Toole GA, & Gitai Z (2014) Surface attachment induces Pseudomonas aeruginosa virulence. *Proceedings of the National Academy of Sciences of the United States of America* 111(47):16860-16865.
17. 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. *Molecular microbiology* 79(2):468-483.
18. Tobe T*, et al.* (2005) Dual regulatory pathways integrating the RcsC-RcsD-RcsB signalling system control enterohaemorrhagic Escherichia coli pathogenicity. *Molecular microbiology* 58(1):320-333.
19. Knutton S, Baldwin T, Williams PH, & McNeish AS (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic Escherichia coli. *Infection and immunity* 57(4):1290-1298.
20. Farfan MJ, Cantero L, Vidal R, Botkin DJ, & Torres AG (2011) Long polar fimbriae of enterohemorrhagic Escherichia coli O157:H7 bind to extracellular matrix proteins. *Infection and immunity* 79(9):3744-3750.
21. Padavannil A*, et al.* (2013) Structure of GrlR-GrlA complex that prevents GrlA activation of virulence genes. *Nature communications* 4:2546.
22. Abe H, Tatsuno I, Tobe T, Okutani A, & Sasakawa C (2002) Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic Escherichia coli O157:H7. *Infection and immunity* 70(7):3500-3509.
23. Tauschek M*, et al.* (2010) Transcriptional analysis of the grlRA virulence operon from Citrobacter rodentium. *Journal of bacteriology* 192(14):3722-3734.
24. Guo P, Weinstein AM, & Weinbaum S (2000) A hydrodynamic mechanosensory hypothesis for brush border microvilli. *American journal of physiology. Renal physiology* 279(4):F698-712.
25. Cairns LS, Marlow VL, Bissett E, Ostrowski A, & Stanley-Wall NR (2013) A mechanical signal transmitted by the flagellum controls signalling in Bacillus subtilis. *Molecular microbiology* 90(1):6-21.
26. Thomas WE, Trintchina E, Forero M, Vogel V, & Sokurenko EV (2002) Bacterial adhesion to target cells enhanced by shear force. *Cell* 109(7):913-923.
27. Tchesnokova V*, et al.* (2010) Shear-enhanced binding of intestinal colonization factor antigen I of enterotoxigenic Escherichia coli. *Molecular microbiology* 76(2):489-502.
28. Makino K*, et al.* (1998) Complete nucleotide sequences of 93-kb and 3.3-kb plasmids of an enterohemorrhagic Escherichia coli O157:H7 derived from Sakai outbreak. *DNA research : an international journal for rapid publication of reports on genes and genomes* 5(1):1-9.
29. Lee DJ*, et al.* (2009) Gene doctoring: a method for recombineering in laboratory and pathogenic Escherichia coli strains. *BMC microbiology* 9:252.
30. 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 microbiology letters* 55(1-2):221-225.
31. Chang AC & Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of bacteriology* 134(3):1141-1156.

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