



Flagellum-Mediated Mechanosensing and RfIP Control Motility State of Pathogenic *Escherichia coli*

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ABSTRACT Bacterial flagellar motility plays an important role in many processes that occur at surfaces or in hydrogels, including adhesion, biofilm formation, and bacterium-host interactions. Consequently, expression of flagellar genes, as well as genes involved in biofilm formation and virulence, can be regulated by the surface contact. In a few bacterial species, flagella themselves are known to serve as mechanosensors, where an increased load on flagella experienced during surface contact or swimming in viscous media controls gene expression. In this study, we show that gene regulation by motility-dependent mechanosensing is common among pathogenic *Escherichia coli* strains. This regulatory mechanism requires flagellar rotation, and it enables pathogenic *E. coli* to repress flagellar genes at low loads in liquid culture, while activating motility in porous medium (soft agar) or upon surface contact. It also controls several other cellular functions, including metabolism and signaling. The mechanosensing response in pathogenic *E. coli* depends on the negative regulator of motility, RfIP (YdiV), which inhibits basal expression of flagellar genes in liquid. While no conditional inhibition of flagellar gene expression in liquid and therefore no upregulation in porous medium was observed in the wild-type commensal or laboratory strains of *E. coli*, mechanosensitive regulation could be recovered by overexpression of RfIP in the laboratory strain. We hypothesize that this conditional activation of flagellar genes in pathogenic *E. coli* reflects adaptation to the dual role played by flagella and motility during infection.

IMPORTANCE Flagella and motility are widespread virulence factors among pathogenic bacteria. Motility enhances the initial host colonization, but the flagellum is a major antigen targeted by the host immune system. Here, we demonstrate that pathogenic *E. coli* strains employ a mechanosensory function of the flagellar motor to activate flagellar expression under high loads, while repressing it in liquid culture. We hypothesize that this mechanism allows pathogenic *E. coli* to regulate its motility dependent on the stage of infection, activating flagellar expression upon initial contact with the host epithelium, when motility is beneficial, but reducing it within the host to delay the immune response.

KEYWORDS *Escherichia coli*, bacterial physiology, flagellar gene regulation, flagellar motility, mechanosensing, virulence

Sensing and rapid adaptation to changing environmental conditions are a common survival strategy of bacteria. One of the best-studied examples of a highly regulated cellular function is flagellum-mediated motility. In *E. coli*, which can exist in various environments and has adopted both commensal and pathogenic lifestyles, the expression of flagellar genes is organized in a hierarchical cascade of three classes that integrate a number of external and internal signals (1, 2). The class I transcriptional regulator FlhDC induces the expression of class II genes, which encode the components of the flagellar hook and basal body as well as a sigma factor, FliA. In turn, FliA is

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TABLE 1 *E. coli* isolates used in this study

<i>E. coli</i> strain ^a	Source of isolation or type of pathogenicity ^b	Motility on TBA 0.27%	Motility in TB ^c
U5/41	UPEC	–	–
DSM 50902	NA	+	–
QST 40139	Possibly commensal strain	–	–
AMC 198	NA	–	–
E611	EPEC	+	–
E2808	EPEC	+	–
EW2129-54	EPEC	+	+/-
C771	EPEC	+	–
ICB 4004	Urine	–	–
S13	Septicemia patient	+	–
IHE3035	Newborn with meningitis	+	+/-
<i>M185/1-1</i>	Fecal isolate from healthy individual	+	+
<i>B185/29-10</i>	Fecal isolate from healthy individual	+	+
D699(U5/41 Oac⁻)	Patient with urinary tract infection	+	–
IHE3043	Newborn with meningitis	–	–
Z36	Patient with urinary tract infection	+	–
T111	Patient with urinary tract infection	+	–
PC 0886	Possibly commensal strain	–	–
CDC 5624-50	EPEC	–	–
<i>MG1655</i>	Commensal lab strain	+	+

^aPathogenic and commensal strains that were motile on TBA 0.27% plates are indicated with boldface and italics, respectively.

^bUPEC, uropathogenic *E. coli*; EPEC, enteropathogenic *E. coli*; NA, not available.

^c+/- indicates that only a fraction of cells are motile.

required for the expression of class III genes, which include those for the outer part of flagella and components of the chemotaxis pathway.

Flagella and motility have several functions in bacterial virulence (3, 4). In the early phase of infection, motility is important for moving through the mucus layer and establishing initial contact with the host epithelium, and flagella can serve as adhesins. However, flagella are also highly immunogenic, being rapidly recognized by the innate immune system (5). Known strategies to avoid this immune response used by such pathogens as *Salmonella*, *Campylobacter*, and *Helicobacter* rely on stochastic variation in the expression of flagellar genes, due either to genetic changes (6–10) or to changes at the transcriptional level (11–13). The latter regulation in *Salmonella* is mediated by RfIP (formerly called YdiV) (14), which represses *fliA* expression by sequestering the FlhDC complex and targeting it for degradation in a subpopulation of cells (15, 16). Expression of RfIP and hence of the fraction of nonmotile cells in the population depends on nutrient levels and cell envelope stress (15, 17).

Although flagellar systems of *E. coli* and *Salmonella* and their regulation are highly similar, only a few *E. coli* strains exhibit genetic phase variation in flagellar expression (18, 19). Furthermore, although the *rflP* gene is present in *E. coli*, it is inactive in laboratory strains because of attenuated translation (20). Thus, it remained unclear what mechanisms might be used by most pathogenic *E. coli* strains to control motility under conditions that resemble those encountered during infection.

In this work, we investigated the motility of several commensal and pathogenic strains of *E. coli* grown in liquid or in soft agar. We showed that pathogenic, but not commensal, *E. coli* strains activate motility only in a porous medium. The underlying regulation requires flagellar rotation as well as expression of RfIP, and it is likely to represent a novel mechanism of motility-dependent mechanosensing.

Pathogenic isolates of *E. coli* downregulate flagellar gene expression in liquid.

We investigated the motility of a number of pathogenic, as well as a few commensal, natural isolates of *E. coli* obtained from the Leibniz Institute's DSMZ collection (Table 1 and see Table S1 in the supplemental material). Surprisingly, while most tested strains (17 out of 19) appeared nonmotile or poorly motile when cultivated in liquid tryptone broth (TB), they became motile on soft (0.27% agar) TB (TBA 0.27%) motility plates, where *E. coli* cells can spread by swimming through agar pores (Table 1; Fig. 1A and B).

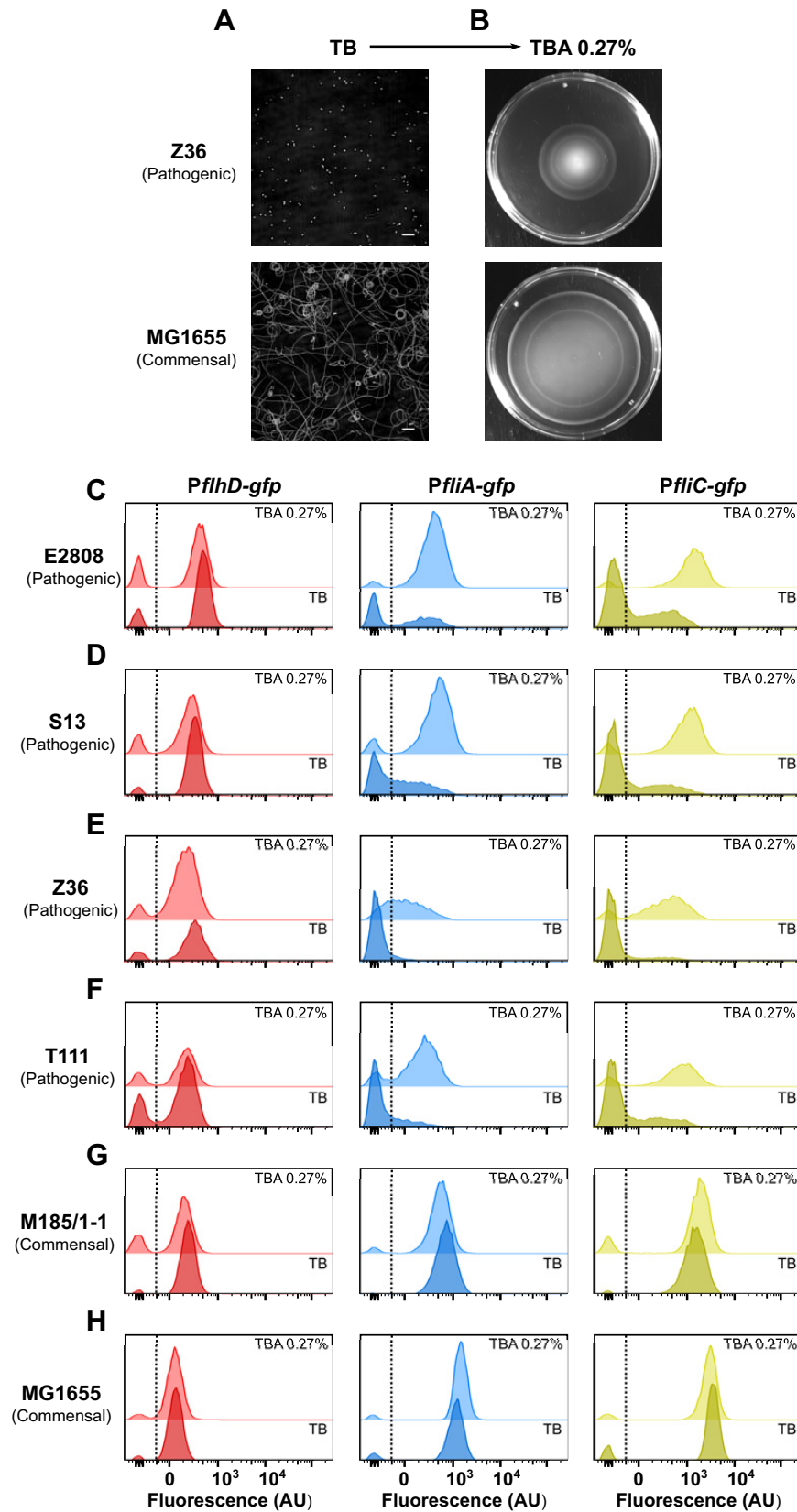


FIG 1 Motilities of pathogenic and commensal *E. coli* isolates in liquid and in 0.27% agar. (A) Cell trajectories of *E. coli* Z36 and MG1655, shown as their projected intensities during 20-s-long movies. Bacteria were grown in liquid TB at 37°C with shaking to an OD₆₀₀ of 0.5 to 0.6, diluted to an OD₆₀₀ of 0.05 to 0.1 in TB, and imaged at room temperature. Scale bars, 50 μm. (B) Motility halos formed by *E. coli*

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Such dependence of motility on growth in agar was confirmed by reinoculation of liquid TB cultures from TBA 0.27% plates and *vice versa*. Interestingly, all *E. coli* strains following this motility pattern were implicated in pathogenicity, whereas both tested commensal strains from the DSMZ collection and the common laboratory strain MG1655 were motile under both conditions.

For further analysis of the underlying regulatory mechanism, we transformed four pathogenic and two commensal *E. coli* strains with plasmids carrying green fluorescent protein (GFP) reporters for all three classes of promoters in a flagellar gene expression cascade. Whereas the activities of the *flhD* (class I) reporters were similar between cells grown in TBA 0.27% and in liquid TB for all strains (Fig. 1C to H and Fig. S1A to F), promoters of *fliA* (class II/class III) and *fliC* (flagellin, class III) were strongly downregulated in liquid TB in pathogenic strains (Fig. 1C to F and Fig. S1A to D). In contrast, no changes in flagellar gene expression were observed in commensal strains (Fig. 1G and H and Fig. S1E and F), consistent with their unchanged motility in liquid culture. Together, these results indicate the existence of a lifestyle-specific motility control in *E. coli* acting at the level of class II flagellar gene expression.

Rotation of the flagellar motor under a load is required for activation of flagellar gene expression in pathogenic *E. coli*. Since the most apparent difference between growth in liquid and that in soft agar is the porous structure of agar, which interferes with flagellar rotation, we hypothesized that an increased mechanical load on the flagellar motor might signal flagellar gene expression in pathogenic *E. coli*. Such mechanosensing has indeed been described in several bacterial species (21, 22), although its molecular mechanisms remain poorly understood. In *E. coli*, the load is known to cause posttranslational remodeling of the flagellar motor, increasing the number of force-generating stator units (23), but no gene-regulatory mechanosensing mediated by flagella has been reported so far.

To test our hypothesis, we made *E. coli* Z36 knockout strains that lack flagellar filaments and therefore have largely reduced motor loads ($\Delta fliC$ mutant) or have paralyzed flagella due to the absence of the MotA stator protein ($\Delta motA$ mutant). These strains were locked in the motility-off state at the level of gene expression in both liquid and agar (Fig. 2A to C; Fig. S2A to C), suggesting that in pathogenic *E. coli*, rotation of the flagellar motor under a load is indeed perceived as a signal activating the expression of class II and class III flagellar genes. These knockouts could be complemented for both motility and gene expression when respective proteins were produced from a plasmid (Fig. S3).

Besides swimming in porous (such as agar) or viscous media, the mechanical load on the flagellar motor is also expected to increase when bacteria swim toward or attach to a surface. Such surface sensing may indeed be the major function of flagellum-mediated mechanosensing (21, 24). Consistently, we observed that the expression of *fliC* became elevated when wild-type (WT) *E. coli* Z36 cells were either forced to the surface by centrifugation (Fig. 2H) or grown on the surface of a semisolid (0.5%) agar (Fig. S4). No *fliC* gene induction was observed in $\Delta motA$ cells under these conditions, suggesting that induction is mediated by the same mechanosensing mechanism. Notably, when cells were grown on a 0.5% agar surface, the *flhD* promoter activity of $\Delta motA$ cells was the same as that of WT *E. coli* Z36 cells (Fig. S4). This confirms that *motA* deletion has no direct effect on *flhD* activity and suggests that the lower expression of *flhD* in nonmotile knockout strains grown on TBA 0.27% than in TB (Fig. 2C and D) was rather due to a high cell density and a corresponding nutrient depletion within the nonspreading colony.

FIG 1 Legend (Continued)

Z36 and MG1655 after 5 μ l of the cells grown in TB (as for panel A) was spotted onto the surface of 0.27% TB agar (TBA 0.27%) and incubated at 37°C for 6 to 8 h. (C to H) Distributions of fluorescence levels of *PflhD-gfp*, *PfliA-gfp*, and *PfliC-gfp* reporters in *E. coli* E2808 (C), S13 (D), Z36 (E), T111 (F), M185/1-1 (G), and MG1655 (H) cells taken from the edges of spreading populations grown in TBA 0.27% or in liquid TB medium, as indicated. Fluorescence was measured using flow cytometry as described previously (46). AU, arbitrary units.

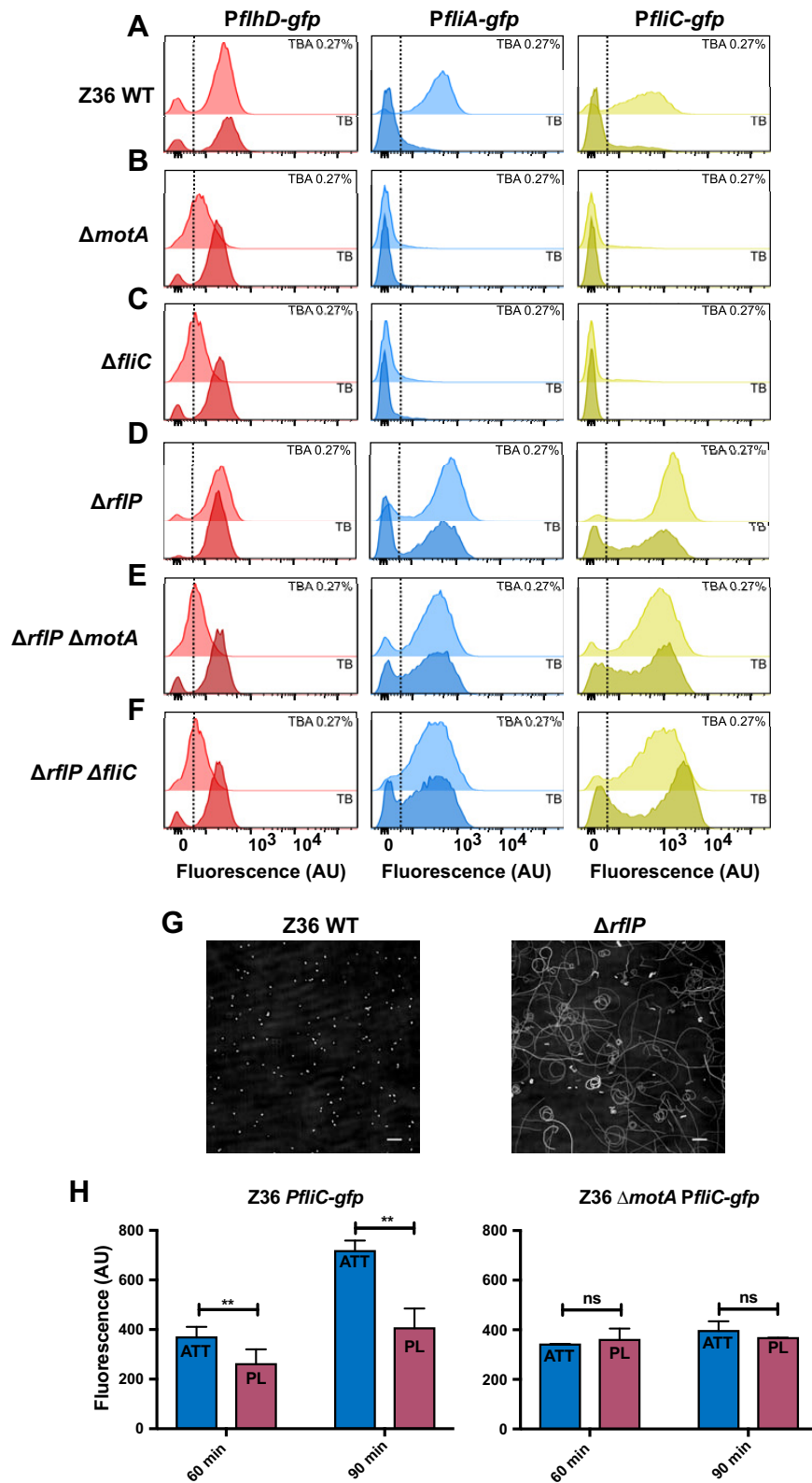


FIG 2 Flagellar motor rotation under a load and RfIP control motility in pathogenic *E. coli*. (A to D) Distributions of fluorescence levels of *PflhD-gfp*, *PfliA-gfp*, and *PflfC-gfp* reporters in wild-type strain *E. coli* Z36 (A) and in its $\Delta motA$ (B), $\Delta fliC$ (C), $\Delta rfIP$ (D), $\Delta rfIP \Delta motA$ (E), and $\Delta rfIP \Delta fliC$ (F) knockout mutants. Bacterial growth and measurements were as described for Fig. 1C to H. (G) Cell trajectories of *E. coli* Z36 and its $\Delta rfIP$ mutant grown in liquid TB medium, acquired as described in the legend of Fig. 1A. Scale bars, 50 μm . (H) *PflfC-gfp* expression in WT Z36 and $\Delta motA$ cells forced to the surfaces of microtiter plates

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Anti-FlhC₂FlhD₄ factor RflP is involved in mechanosensing. Since *fliA* and *fliC* are known to be repressed by RflP in *Salmonella*, we tested the effect of *rflP* deletion on the observed regulation. Unlike in the laboratory *E. coli* strains where RflP had no effect on flagellar gene expression (20), *rflP* deletion in *E. coli* Z36 resulted in strong derepression of *fliA* and *fliC* expression, particularly apparent in liquid TB medium (Fig. 2D and Fig. S2D). Consequently, most *E. coli* Z36 $\Delta rflP$ cells remained motile in liquid (Fig. 2G). This effect could be complemented by expressing RflP from the plasmid (Fig. S5). Deletion of *rflP* also derepressed the flagellar gene expression in the $\Delta motA$ and $\Delta fliC$ backgrounds, both in liquid and on TBA 0.27% (Fig. 2E and F and Fig. S2E and F). Although *fliA* and particularly *fliC* expression was still weakly upregulated on soft agar even in *E. coli* Z36 $\Delta rflP$, it remains unclear whether this residual RflP-independent regulation is due to mechanosensing, since to some extent it was also observed in $\Delta rflP$ $\Delta motA$ and $\Delta rflP$ $\Delta fliC$ double knockouts (Fig. 2E and F and Fig. S2E and F).

The interplay between RflP-mediated repression and mechanosensing remains to be elucidated (see below). Nevertheless, we observed that the activity of the *rflP* promoter was independent of the cultivation conditions (Fig. S6), suggesting that RflP is not regulated by mechanosensing at the transcriptional level. This is in contrast to the transcriptional regulation of *rflP* by cell envelope stress in *Salmonella* (17).

Importantly, our results also indicate that the determinants of mechanosensing other than RflP are present in *E. coli* MG1655. The overexpression of *rflP* not only led to the expected dose-dependent inhibition of *fliC* expression in MG1655 (Fig. 3A) but also apparently restored mechanosensing in this laboratory strain, with elevated *fliC* expression in soft agar compared to that in liquid (Fig. 3B). Since the inhibition of *fliC* expression by RflP was only partial in MG1655, this increase was particularly pronounced at the level of mean reporter activity rather than as a fraction of positive cells.

Proteome regulation by mechanosensing. In order to identify nonflagellar proteins that could be regulated by mechanosensing, we performed comparative whole-proteome analysis of WT *E. coli* Z36 and $\Delta motA$ cells grown either on 0.5% agar or in liquid TB medium. Apart from flagellar motor proteins, several metabolic enzymes and uncharacterized proteins were upregulated in a *motA*-dependent fashion on the agar surface, whereas several transporters and metabolic enzymes, as well as the sensor kinase QseC, were downregulated (Table 2). Since RflP could not be detected in these samples, its regulation at the protein level could not be assessed. Moreover, the expression of many other proteins, some of them involved in the acid stress response, was upregulated on the surface in a *motA*-independent manner (Table S2). Since QseC, a member of the QseB QseC two-component regulatory system, was among the most strongly downregulated proteins and it was previously shown to play a role in the regulation of flagellar expression (25, 26), we tested whether this system might have a regulatory role in mechanosensing. However, no effect of *qseC* deletion on *fliC* expression could be observed in *E. coli* Z36, grown either on soft agar or in TB (Fig. S7), suggesting that under our conditions and in this strain, this two-component system is inactive. Thus, QseC does not seem to have a mechanosensory role. Nevertheless, its mechanosensitive regulation by pathogenic *E. coli* might be important during infection, since QseC was shown to be important for virulence (26, 27).

In this study, we demonstrate that the expression of flagellar genes in a number of pathogenic *E. coli* strains is inhibited in liquid but that their expression is induced when bacteria are grown either in porous medium or on a surface. Because this regulation was not observed in commensal or laboratory strains of *E. coli*, it likely represents a specific mechanism to avoid the adaptive immune response of the host, akin to genetic or transcriptional variation in the expression of flagellar genes observed in other

FIG 2 Legend (Continued)

(attached [ATT]) by centrifugation or statically incubated (planktonic [PL]), measured by flow cytometry. The mean fluorescence of each GFP-positive subpopulation is shown in arbitrary units (AU). Means from a minimum of four independent replicas are shown; error bars represent standard deviations. *P* values were calculated using the Mann-Whitney test. **, *P* < 0.005; ns, not significant.

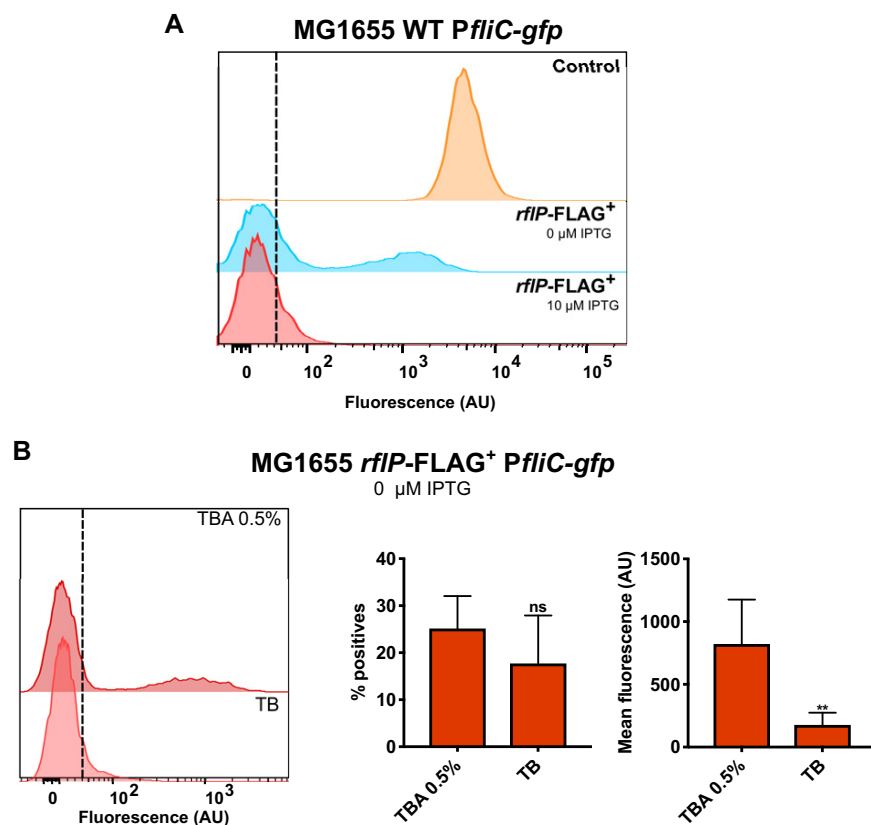


FIG 3 The expression of RflP restores mechanosensing in *E. coli* MG1655. (A) Distribution of fluorescence levels of *PflC-gfp* in *E. coli* MG1655 expressing an *rflP-FLAG* fusion from an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter, without induction or induced with 10 μ M IPTG. Cells were grown in liquid TB as described for Fig. 1. (B) Distribution of fluorescence levels of *PflC-gfp* in MG1655 cells expressing RflP, grown on TBA 0.5% or in liquid TB. Media were supplemented with ampicillin to prevent plasmid loss. Fluorescence was quantified as described for Fig. 1. Means from a minimum of seven independent replicates are shown; error bars represent standard deviations. *P* values were calculated using the Mann-Whitney test (**, $P < 0.005$; ns, not significant).

bacterial pathogens (6–12, 28). However, unlike with the stochastic antigenic variation or transcriptional switching observed in these bacteria, the control of flagellar gene expression in pathogenic *E. coli* apparently relies on sensing the mechanical load on the flagellar motor.

Although surface sensing-mediated control of gene expression, including control of bacterial flagellation, was reported before, most notably in *Vibrio*, *Caulobacter*, and *Bacillus* species (24, 29–35), this is, to our knowledge, the first example of such regulation in *E. coli*. It was previously shown that enterohemorrhagic *E. coli* (EHEC) can regulate the expression of EHEC-specific virulence genes upon sensing host attachment and fluid shear (36), but this surface sensing is not known to rely on flagella or to regulate motility. Moreover, this gene-regulatory mechanosensing controls motility in a different way than was previously described for posttranslational remodeling of the *E. coli* flagellar motor under a mechanical load (23, 37).

We hypothesize that such motility-dependent mechanosensing allows pathogenic *E. coli* to express flagella upon contact with epithelial cells or in mucus when motility is beneficial for the initial attachment to the host but that pathogenic *E. coli* shuts down the expression of flagellar genes upon entry into the host in order to delay detection by the immune system. In line with apparent trade-offs of flagellin expression in pathogenic *E. coli*, we also observed bimodal flagellar expression in these strains, with a subpopulation of cells remaining in the flagellum-off state even in soft agar. A similar bimodal expression was previously observed in other bacteria, including *Salmonella*

TABLE 2 Proteins regulated by motility-dependent surface sensing in *E. coli* Z36^a

Protein	Function	Fold change between:		
		$\Delta motA_{surf}$ and WT _{surf}	WT _{liq} and WT _{surf}	$\Delta motA_{liq}$ and $\Delta motA_{surf}$
YhdP	Uncharacterized protein	-21.18	-177.91	-1.13
IroE	Uncharacterized protein	-5.49	-78.78	1.60
PrpC	Citrate synthase	-31.93	-24.14	1.13
PrpD	2-Methylcitrate dehydratase	-4.96	-5.62	-1.03
PrpE	Propionate-CoA ligase	-3.92	-5.34	-1.21
FliH	Flagellar assembly protein	-3.71	-2.22	1.77
FliS	Flagellar protein	-3.20	-2.10	1.88
ElaB	Uncharacterized protein	7.66	-1.61	-2.15
MotB	Flagellar stator component	-5.02	-1.27	2.75
UbiB	Probable protein kinase	29.56	1.00	-1.25
YfiQ	Uncharacterized protein	7.61	2.32	-1.52
UidR	HTH-type transcriptional regulator	3.65	2.51	-1.63
C2460	Putative polyketide synthase	3.56	3.42	1.64
TdcF	Putative reactive intermediate deaminase	3.34	3.54	1.09
AnmK	Anhydro-N-acetylmuramic acid kinase	3.10	3.75	-1.15
YffB	ArsC family protein	4.44	4.07	1.81
Gst	Glutathione S-transferase	4.05	4.78	1.19
Alr	Alanine racemase, biosynthetic	4.60	5.11	1.19
ThiQ	Thiamine import ATP-binding protein	4.81	5.46	1.10
NrdG	Anaerobic ribonucleoside-triphosphate reductase-activating protein	5.57	6.79	1.19
SdhE	FAD assembly factor	5.20	7.30	-1.88
MobB	Molybdopterin-guanine dinucleotide biosynthesis protein	4.95	7.76	2.42
C3307	Putative conserved protein	5.65	10.05	1.15
KefC	Glutathione-regulated potassium efflux system protein	22.47	10.50	-1.53
YheV	Uncharacterized protein	4.34	11.64	1.94
C1760	Putative transcriptional repressor	19.74	13.14	-1.12
SapA	Peptide transport periplasmic protein	19.54	18.54	1.46
NusB	N utilization substance protein	17.48	20.27	1.60
YddE	Uncharacterized protein	29.57	21.36	-1.88
C4017	Putative ribose ABC transporter	6.30	22.17	2.92
YjjU	Uncharacterized protein	77.64	37.40	-1.68
YbbL	Hypothetical ABC transporter ATP-binding protein	26.50	43.96	2.04
YcjZ	Hypothetical transcriptional regulator	5.53	63.19	1.70
IdnK	Gluconokinase	33.19	84.34	2.27
ThiJ	4-Methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	163.82	111.05	-1.09
CyoC	Cytochrome <i>bo</i> ₃ ubiquinol oxidase subunit 3	69.54	130.08	1.47
YgiY	Uncharacterized protein	103.01	132.15	1.01
QseC	Sensor protein	103.01	132.15	1.01
RlmC	23S rRNA [uracil ⁷⁴⁷ -C ⁵]methyltransferase	92.80	166.17	2.17

^aProteins were selected from the whole-proteome data based on the following criteria: differential expression between the WT and the $\Delta motA$ mutant (fold change, ≥ 3) on TBA 0.5%, differential expression between the WT on TBA 0.5% (WT_{surf}) and in liquid TB (WT_{liq}), and little or no difference (fold change, < 3) between the $\Delta motA$ mutant on TBA 0.5% ($\Delta motA_{surf}$) and in liquid TB ($\Delta motA_{liq}$). The *E. coli* CFT073 protein annotation database was used as a reference. CoA, coenzyme A; HTH, helix turn helix; FAD, flavin adenine dinucleotide.

(16) and *Bacillus subtilis* (38), but whereas in *Salmonella* bimodal *fliC* expression is known to depend on RfIP activity (16, 17), deletion of *rflP* in *E. coli* Z36 did not abolish the bimodality of *fliC* expression, suggesting a different origin of bimodality.

Though the underlying mechanism of signal transduction from the flagellar motor to transcriptional regulation remains to be established, it apparently requires flagellar rotation under a load. The motility-off state of gene expression was observed not only for the filament-deficient $\Delta fliC$ strain of *E. coli* that cannot efficiently sense the load but also for the $\Delta motA$ strain, which has immobilized flagella. This contrasts with observations of *B. subtilis* and *Vibrio parahaemolyticus*; these organisms with deletions of motor proteins mimic strains under high loads (30, 32), but their phenotype is similar to the lock-off mechanosensing phenotype of *mot* mutants of *Caulobacter crescentus* and *Pseudomonas aeruginosa* (24, 39).

Our results demonstrate that mechanosensing occurs in pathogenic *E. coli* strains due to the activity of the motility repressor RflP, which in these strains inhibits the expression of flagellar genes in liquid. Consistently, artificial upregulation of RflP in *E. coli* K-12 unmasks its mechanosensing response, demonstrating that other sensory determinants are present in the laboratory strain. While at this stage it cannot be ruled out that RflP-stimulated proteolysis of the FlhDC complex is required only to generally lower the basal expression of flagellar genes, its involvement in mechanosensing might also be direct, as occurred with a previously reported surface contact-dependent relief of proteolysis of a master flagellar regulator in *B. subtilis* (31).

Bacterial strains and culture conditions. All strains and plasmids used in this study are listed in Table S1. *E. coli* strains were grown in liquid tryptone broth (TB) medium (10 g tryptone and 5 g NaCl per liter) or in lysogeny broth (LB) medium (10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) supplemented with antibiotics where necessary.

Cloning and knockout strain construction. In order to construct the *rflP-gfp* fluorescent reporter, the bp -253-to-bp +90 promoter region of the *rflP* gene was amplified from the *E. coli* Z36 chromosome and cloned into the pUA66 vector using XhoI and BamHI restriction sites (40). For *rflP*-FLAG plasmid construction, the *rflP* gene sequence without a stop codon was amplified from the *E. coli* Z36 chromosome with a FLAG coding sequence, with the stop codon being added via a primer overhang. The resulting fusion construct was cloned into the pTrc99A vector using SacI and XbaI restriction sites (41).

All the knockout strains described in this work were obtained using the lambda red recombination system as described previously (42). Where necessary, kanamycin cassettes were flipped out using FLP-FLP recombination target (FRT) recombination (43).

Motility assay in soft agar. Single colonies of different *E. coli* strains were transferred onto the surfaces of soft agar plates (TB containing 0.27% agar), and the motile behavior of *E. coli* strains was assessed after 6 to 8 h of incubation at 37°C by analyzing the motility halo diameter. The cells from the edges of such halos were subsequently transferred into 3 ml of fresh TB medium and grown with shaking at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6. The motility of the cells was then analyzed using phase-contrast microscopy.

Single-bacterium movement tracking. *E. coli* MG1655, Z36, and Z36 $\Delta rflP$ overnight cultures were diluted in fresh TB and grown at 37°C with shaking to the OD_{600} of 0.6. The samples were subsequently diluted to final OD_{600} s of 0.05 to 0.1 in TB and loaded into ibidi channels (μ -Slide Chemotaxis^{3D}; ibidi GmbH, Germany). Movies were recorded at $\times 10$ magnification with a phase-contrast microscope at a rate of 20 frames per second using a complementary metal-oxide semiconductor (CMOS) camera (exposure time, 1 ms), normalized by dividing each frame by an image of the background illumination under the same conditions and inverted in order for the cells to appear bright. A projection of the temporal maximum of each pixel was computed to produce an image of the cell trajectories.

Flow cytometry. Promoter activities of the flagellar genes *flhD*, *fliA*, and *fliC* and the motility regulator *rflP* were assayed using plasmid-based reporters containing the respective promoter regions fused to *gfp* (Table S1). Promoter activities were analyzed in the cells collected from the edges of motility halos formed on 0.27% TBA plates and after subsequent cultivation in liquid TB medium. The samples were diluted in 2 ml tethering buffer (10 mM KH_2PO_4 , 100 μ M EDTA, 1 μ M L-methionine, and 10 mM sodium lactate, pH 7.0), and fluorescence was measured with a BD LSRFortessa SORP cell analyzer (BD Biosciences, Germany).

In order to compare *PfliC-gfp* activity in attached and planktonic cells, 300 μ l of WT Z36 and $\Delta motA$ mutant day cultures in TB ($OD_{600} = 0.6$, diluted in fresh TB to the final OD_{600} of 0.05) were inoculated into the wells of a 96-well microtiter plate (ibidi, Germany), and the cells were centrifuged for 45 min (37°C, 4,000 rpm). The supernatant with nonattached planktonic cells was transferred into new wells. The wells with

attached cells were washed three times with 500 μ l tethering buffer, and the presence of attached cells was verified with phase-contrast microscopy. Fresh TB (300 μ l) was subsequently added to the wells, and the plates were incubated at 37°C. The measurements of *Pfl*C-*gfp* activity in attached and planktonic cells were performed with flow cytometry after 60 and 90 min of incubation.

Proteomic analysis. To compare the proteomes of WT *E. coli* Z36 and Δ *motA* strains grown on the surfaces of soft agar plates and in the liquid medium, the cells were grown on the surface of 0.5% TBA for 4 h at 37°C. Flagellar gene expression was confirmed with flow cytometry using *Pfl*C-*gfp* promoter fusion activity as a readout. The cells were then washed off the plates with 2 ml cold phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄, 0.27 g KH₂PO₄ per liter), and the final sample size was adjusted to 5 ml with an OD₆₀₀ of 0.6. The cells were then washed twice in 5 ml cold PBS, and the pellets were stored at –20°C. Before the samples were frozen, 50 μ l of the suspension was reinoculated into 5 ml of liquid TB and grown at 37°C with shaking to an OD₆₀₀ of 0.6. The cells were then washed twice in 5 ml cold PBS, and the pellets were stored at –20°C. The collected samples were subsequently subjected to proteomic analysis using mass spectrometry and subsequent data analysis as described before (44, 45). Although no reference database for *E. coli* Z36 is available, using the *E. coli* CFT073 protein database resulted in full coverage of the analyzed proteome.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 0.1 MB.

FIG S2, EPS file, 0.2 MB.

FIG S3, PDF file, 0.1 MB.

FIG S4, EPS file, 0.1 MB.

FIG S5, EPS file, 0.1 MB.

FIG S6, EPS file, 0.1 MB.

FIG S7, EPS file, 0.1 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.02 MB.

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L.L., M.E.L., R.C., and V.S. designed the experiments. L.L., M.E.L., and R.C. performed the experiments and analyzed the data. L.L. and V.S. wrote the manuscript.

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