The *Proteus mirabilis rffG* gene is essential for swarm-associated cell elongation through an

Rcs-mediated pathway

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Abstract

The gram-negative bacterium *Proteus mirabilis* can elongate up to 40-fold on surfaces before engaging in a social motility termed "swarming" that precedes division into short non-motile rod-shaped cells. The specific physical and genetic requirements for swarmer cell elongation are not fully understood. Here we further elucidate critical components for swarmer cell elongation. We show that the gene, *rffG,* is essential for cell envelope structure and integrity on a surface. The *rffG* gene encodes a predicted biosynthesis protein for the cell-envelope component, Enterobacterial Common Antigen. We observed that the production of large LPS-linked moieties requires the *rffG* gene; the chemical properties of these moieties remain unknown, but their loss corresponded with inhibition of swarmer cell elongation. We further show that inhibition of elongation occurs through the actions of the Rcs phosphorelay. The Rcs phosphorelay, which responds to cell envelope stress, is a known repressor of the master transcriptional regulator, $FlhD_4C_2$. Loss of *rffG* and the Rcs response regulator rescued motility but induced severe cell shape defects. These observations indicated that though swarmer motility and elongation into swarmer cells are genetically linked through Rcs, these are distinct processes during swarming. Building upon consensus models, we hypothesize that the Rcs phosphorelay in *P. mirabilis* functions as a developmental checkpoint to prevent cell envelope-compromised cells from initiating swarmer cell elongation and subsequent motility.

Importance statement

Proteus mirabilis is a primary cause of catheter-associated urinary tract infections, and its surface-based swarm motility has been implicated in virulence. Here we have characterized a

45   novel essentiality of the *rffG* gene in producing large LPS-linked moieties needed for cell

46   elongation, which in itself is a necessary first step of swarming. We have also elucidated a

47   previously undescribed role for the conserved Rcs stress response: retention of cell shape

48   separate from inhibition of the *flhDC* flagellar regulon. These data contribute to a broader

49   understanding of Rcs as a multifunctional regulator of cell integrity, motility, and virulence.

Introduction

Many bacteria move along a surface using a group motility termed swarming. For *Proteus mirabilis*, a gram-negative commensal member of the human gut microbiome and opportunistic pathogen, rapid surface based swarm motility likely contributes to its virulence during catheter associated urinary tract infections (1, 2). *P. mirabilis* cells initiate a developmental cell cycle upon surface growth. Cells elongate from 1-2 μm rods into hyper-flagellated, snake-like "swarmer" cells that can range from 10 – 80 μm in length (3, 4). Swarmer cells contain multiple chromosomes, are extensively flagellated, and closely associate into multi-cell rafts that collectively move across a surface (3, 4). After a defined period of motility, swarmer cells divide into short (1-2 μm) non-motile rod-shaped cells containing one chromosome (5). Iterative rounds of swarmer cell elongation, group motility, and cell division result in the rapid occupation of centimeter-scale surfaces in a stereotypical concentric ring pattern (5). Elongation into swarmer cells is coordinated with changes to the cell envelope, most notably in the alteration of outer membrane (OM) structure and properties: the lipopolysaccharide (LPS) structure changes, OM fluidity increases, and areas of lipid bilayer arise in the outer membrane (6-8). Cells also transition through unknown mechanisms from being short and rigid to long and flexible with a phenotypic S-shaped curving (3, 4), suggesting uncharacterized changes to cell wall structure may occur.

Cell envelope structure and stress sensing appear to play broadly conserved roles in swarm regulation in several bacterial species, including *P. mirabilis*, *Escherichia coli*, and *Serratia marcescens* (9-17). Interrogating the contribution of specific aspects of cell envelope structure to cell elongation has proven challenging. The three broad cell envelope biosynthesis pathways in Enterobacteriaceae produce LPS, Enterobacterial Common Antigen (ECA), and

peptidoglycan (PG) for the cell wall. In *E. coli*, genetic modifications to each of these

biosynthetic pathways can dramatically alter cell shape and motility due to perturbations in the

balance of cell envelope substrates that are shared between the pathways (12, 13). For example,

genetic deletion of specific LPS and ECA biosynthesis genes results in the accumulation of dead-

end intermediate products that sequester undecaprenyl phosphate (12, 13). In addition,

perturbation of cell wall biosynthesis and formation of inert peptidoglycan islands can lead to

cell division and branching defects in other bacterial species (18-21).

Strikingly, such defects are not observed in swarming *P. mirabilis* even though the cells

regularly oscillate between being short and rigid versus long and flexible. How *P. mirabilis* cells

can withstand regular dramatic changes in cell envelope structure during swarmer cell elongation

is not fully explained. For example, the effects of perturbing LPS or ECA biosynthesis in *P.*

mirabilis are not resolved. Disruption of LPS biosynthesis genes *waaL* (15, 22) or *ugd* (23)

inhibits swarmer cell elongation and motility through inhibition of surface sensing (22) and

activation of RpoE (16, 23), respectively. However, disruption of ECA ligation through deletion

of *wzyE* has minimal effects on *P. mirabilis* elongation or motility (22). In this paper, we address

whether disruption of the ECA biosynthesis pathway upstream of the *wzyE*-encoded ligase

affects swarming, and further, how cells respond to these cell envelope perturbations.

Across several bacterial species, the Rcs phosphorelay has been directly implicated in

swarm regulation; this phosphorelay functions by altering gene expression via the transcriptional

response regulator, RcsB (9, 10, 15, 24) (see also reviews (25, 26)). RcsB serves as a potent

inhibitor of swarmer cell elongation and motility by blocking expression of the genes *flhDC*,

which encode the master transcriptional regulator of swarming $FlhD_4C_2$ (10, 14). $FlhD_4C_2$ is an

early activator of flagellar gene expression in *P. mirabilis*, *E. coli*, and other bacteria species.

Release of Rcs inhibition is sufficient to drive swarmer cell development; RcsB-deficient *P.*

mirabilis cells constitutively elongate in liquid (14). Multiple cell envelope stresses, such as

perturbations to ECA or LPS, activate the Rcs response in several bacterial species (9, 15, 24).

Less clear is whether the Rcs response to cell envelope stress during swarming acts solely

through the *flhDC* regulon. The Rcs regulon in *P. mirabilis* was recently defined, and

intriguingly, RcsB directly modulates expression of cell division genes to regulate swarmer cell

development independently of $FlhD_4C_2$ (27, 28). This result is consistent with earlier findings in

E. coli: Rcs has conditional roles in *de novo* cell shape establishment (29). Therefore, we also

assessed whether the cell response to membrane perturbations proceeds simply and directly

through the predicted Rcs and $FlhD_4C_2$ pathways.

Here we present evidence that cell envelope integrity, produced in part by the *rffG* gene,

is essential for swarmer cell elongation and that Rcs is necessary for maintaining cell shape in

pathways separate from $FlhD_4C_2$-regulated elongation and motility. We show that *P. mirabilis*

cells require the ECA biosynthesis gene *rffG* to produce uncharacterized high molecular weight

(HMW) LPS-bound moieties. Loss of these moieties correlated with inhibition of swarmer cell

elongation through the Rcs phosphorelay and *flhDC*. Notably, we elucidated that the Rcs

response to cell envelope defects in *P. mirabilis* encompassed pathways separate from *flhDC*

through genetic analyses and gene expression profiling. We propose that the Rcs phosphorelay

serves as or functions within a developmental checkpoint for cells on swarm-permissive

surfaces. Further, we propose that disruptions in biosynthetic pathways resulting in compromised

cell envelopes activate the Rcs phosphorelay to mediate cell development on surfaces.

Results

The *rffG* gene is essential for the production of LPS-associated OM moieties

We examined the potential roles of less-studied OM components by targeting the ECA

biosynthesis gene, *rffG*, in wild-type *P. mirabilis* (WT). A homologous *rff* gene cluster is

responsible for ECA production in *E. coli* (30). We generated a deletion of the *rffG* gene, which

is predicted to encode a dTDP glucose-4,6-dehydratase; this mutant is hereafter called "Δ*rffG*".

We also complemented Δ*rffG* through expression of *rffG* under the *lac* promoter on a plasmid,

which constitutively expresses in *P. mirabilis* (22); this strain is hereafter called "*rffG*-

complemented."

Understanding of ECA state and localization in *P. mirabilis* is incomplete. ECA exists in

many forms: linked to other lipids, found in a circularized and soluble form, or surface-exposed

and linked to the LPS core (30-35). Therefore to characterize whether the loss of *rffG* alters OM

structures, we extracted LPS from surface-grown colonies of WT, Δ*rffG,* and *rffG*-complemented

populations and visualized the LPS-associated moieties using silver stain (36). The observed

banding patterns of the WT and *rffG*-complemented populations were nearly equivalent (Figure

1A). The banding pattern of the Δ*rffG* population, however, lacks a high molecular weight

(HMW) smear, and the bands within the putative O-antigen ladder formed doublets instead of

singlets (Figure 1A). We concluded that *rffG* is essential for production of wild-type O-antigen

and the HMW smear. We inferred that the HMW smear might consist of LPS-associated ECA

moieties based on the proposed function of *rffG* in the production of ECA. However, we are

conservatively referencing this smear as "HMW LPS-associated moieties" as opposed to "LPS-

linked ECA" because repeated efforts to confirm the presence of ECA via Western blotting with

E. coli O14 serum (reactive against ECA) (SSI Diagnostica, Hillerød, Denmark) (37, 38) were

unsuccessful.

We additionally predicted that these observed perturbations to the LPS components in the

Δ*rffG* cells likely cause broader cell envelope damage. We assessed cell viability and cell

envelope integrity to address whether Δ*rffG* cells might exhibit broad disruption of cell envelope

structure and/or integrity. We first measured the growth of WT, Δ*rffG*, and *rffG*-complemented

in nutrient-rich broth and on nutrient-rich agar. Growth in either condition was not impacted by

loss of the *rffG* gene (Figure SF1A, SF1B), though the colony size of Δ*rffG* was smaller when

grown on solid agar (Figure SF1C). We next used envelope-challenging conditions to probe the

integrity of the cell envelope. To target the OM, we measured resistance to Polymyxin B (PmB)

and bile salts. PmB is thought to act by targeting the LPS; disruption of LPS biosynthesis genes

induce PmB sensitivity in *P. mirabilis* (23, 39). Bile salts broadly target membranes through

detergent-like effects (40). ECA is likely involved in bile salts resistance of *Salmonella enterica*

(41); however, a role for ECA in bile salt resistance of *P. mirabilis* has not been explored. Both

WT and the Δ*rffG* strains were resistant to 50 mg/mL PmB (Figure SF1D), which approaches the

solubility of PmB in water. Both WT and Δ*rffG* populations grew on 0.1% bile salts and

exhibited decreased growth at 0.2% and 0.5% (Figure 1B). However, the growth defects of Δ*rffG*

populations on 0.2% were relatively more severe than those of WT cells; populations formed

small and translucent colonies (Figure S1B). Therefore, the Δ*rffG* populations exhibited growth

defects in the presence of bile salts as compared to WT, but not in PmB, indicating that the OM

integrity is compromised in the Δ*rffG* cells (Figure 1B).

To further interrogate cell envelope integrity, we analyzed cell morphology in response to

subclinical levels of carbenicillin (10 µg/ml Cb). WT cells lengthened to tens of microns after

two or four hours of Cb treatment, and the overall appearance was even in width with occasional

mid-cell bloating (Figure 1C); this observation is comparable to reports for *E. coli* and *P.*

mirabilis (42). Bloating became more pronounced after four hours of exposure, though little cell

lysis was observed. The Δ*rffG* cells exhibited cell shape defects after one hour of Cb exposure.

These defects became enhanced after two hours as cells elongated, and the majority of cells were

lysed after four hours on Cb (Figure 1C). Equivalent results were attained with Aztreonam, an

inhibitor of the cell division protein, FtsI (Figure SF1E). We deduced that *rffG*-deficient cells

were sensitized to cell wall stress and contained compromised cell envelopes, indicating that the

rffG gene is essential for wild-type cell envelope formation.

Cells require *rffG* to complete swarmer cell elongation and initiate swarming

As perturbations to LPS structure are known to inhibit swarm motility in *P. mirabilis*

(22), we predicted that *rffG*-deficient cells would be prohibited from swarming. We

characterized the swarm developmental cycle of WT, Δ*rffG,* and *rffG*-complemented

populations. Swarms of the WT population occupied a circle of 10-centimeter diameter on rich

media within 24 hours (Figure 2A). Under the same conditions, swarms of the Δ*rffG* population

did not expand beyond the inoculum, while swarms of the *rffG*-complemented populations

expanded roughly half the distance of WT and formed a restricted terrace structure (Figure 2A).

Hence, the *rffG* gene was necessary for swarm colony expansion; however, the native levels or

expression dynamics of *rffG* expression likely contribute to its activity.

It remained unclear at which stage of swarming the Δ*rffG* cells likely inhibited:

elongation and/or motility. We examined populations of WT, Δ*rffG*, and *rffG*-complemented

cells using epifluorescence microscopy under swarm-permissive conditions to assess cell

morphology. A fluorescent Venus reporter downstream of *fliA* was introduced on the

chromosomes of each strain background to visualize flagellar gene expression across each

population. The *fliA* gene is directly regulated by FlhD$_4$C$_2$ and is highly expressed in swarming

cells (43, 44). By four hours after inoculation, WT populations comprised both short non-motile

and elongated motile cells, demonstrating that cells progressed normally through the swarm cell

cycle. Populations became enriched in elongated cells that brightly expressed the *fliA* reporter by

six hours (Figure 2C). These observations confirm previous observations that expression of

flagellar genes occurs in a time-dependent manner on surfaces (22). By contrast, most Δ*rffG* cells

at four and six hours were short, non-motile, and at six hours largely did not exhibit *fliA* reporter-

induced fluorescence (Figure 2C). A subset of Δ*rffG* cells did appear to initiate elongation

(Figure 2C) but often lysed or divided into short cells (Figure SF2). The *rffG*-complemented

cells formed elongated motile cells with *fliA* reporter fluorescence comparable to WT by six

hours, though this progression was delayed compared to WT cells (Figure 2C). Altogether, these

observations indicated that *rffG* is necessary for cells to exhibit two hallmarks of swarmer cell

development: cell elongation and flagellar gene expression. We concluded that the impaired

swarm motility was because *rffG*-deficient cells are unable to elongate into swarmer cells.

***rffG*-deficient cells have a gene expression profile distinct from that of WT**

Inhibition of swarm elongation in *rffG*-deficient cells could be achieved via a physical

defect of the cell membrane or via activation of an inhibitory signaling cascade upstream of

known swarm-promoting factors. LPS deficiencies initiate signaling via the Rcs phosphorelay

and suppress swarming via RcsB inhibition of *flhDC* expression (15). To resolve how swarmer

cell elongation is inhibited in *rffG*-deficient cells, we performed two rounds of RNA-Seq

analysis on WT and Δ*rffG* populations grown in swarm-permissive conditions so that we could

identify pathways disrupted in the Δ*rffG* background. As there are known transcriptional

differences between non-motile and actively swarming cells (44), we used short, non-motile WT

cells harvested from swarms.

We stringently considered "significant" any expression levels which were at least four-

fold divergent from that of WT. Of ~ 3500 genes in the *P. mirabilis* genome, 207 genes were

significantly increased in Δ*rffG* as compared to WT (Table S1, Figure 3A), and of these genes,

about a third could not be assigned a function (Figure 3B). Notably, expression of 29 fimbria or

adhesion related genes was increased; these genes included the virulence-associated MR/P

fimbrial operon (200-fold increased expression of *mrpA*) and *pmpA* (*Proteus* P-like pili, 55.5-

fold increased) (Table 1, Table S1). Several genes related to carnitine metabolism (the

transcriptional regulator *caiF* at 63.6-fold and *caiA, fixC,* and *fixX* at four-fold), as well as genes

related to the TCA cycle and oxidative phosphorylation were also up-regulated, including

frdABCD (4-8 fold increased) and *cydAB* (4-6-fold increased) (Tables 1, Table S1). Carnitine can

be metabolized under aerobic conditions for *Proteus*, but the greatest metabolism occurs under

anaerobic conditions (45, 46). Carnitine metabolism is a nutrient source as well as a stress

protectant for several bacterial species (47-49). Likewise, *ompW* was increased ~ 33.7 fold along

with *dcuB*, an anaerobic C4-dicarboxylate transporter, at 29.6 fold (Table 1, Table S1). In *E. coli*,

maximal *ompW* expression is tied to survival in the transition from aerobic to anaerobic growth

(50). In summary, the Δ*rffG* cells have upregulated genes for fimbrial production and for

metabolism under anaerobic or micro-aerobic environments.

Conversely, we observed decreased expression of 136 genes in the Δ*rffG* populations

relative to WT (Table S2, Figure 3A), of which approximately a third could not be assigned a

function (Figure 3B). 20% of the down-regulated genes were directly related to flagella assembly

or chemotaxis (Tables 2, Table S2). In addition, factors known to promote swarmer cell

development and motility also had decreased expression, including *umoD* at 0.12-fold, *umoA* at

0.19-fold expressed, and *ccm* at 0.12-fold expressed (Table 2). Another approximately 18% of

the down-regulated genes were related to ribosome structure and translation (Figure 3B).

Additionally, three key regulators linked to stress or fimbrial production were differentially

regulated: *mrpJ* (19.1-fold increased) (51), *rpoS* (8.7-fold increased), and the RcsB-cofactor *rcsA*

(9.9-fold increased) (27, 28). Interestingly, we observed partial overlap between the genes

differentially regulated in Δ*rffG* and the recently characterized regulon of RcsB in *P. mirabilis*

(27, 28) (Figure 3A). The overlap was especially significant amongst down-regulated genes

(Figure 3A). RcsB negatively regulates the *flhDC* regulon (flagella and chemotaxis genes) and

positively regulates the *mrpJ* gene (27, 28). We hypothesized that the Rcs phosphorelay was

active in the Δ*rffG* population given this expression profile.

***flhDC* and RcsB respond to Δ*rffG*-associated cell elongation and swarm defects**

Based on a consensus model derived from previous research (15, 24, 52), we posited that

Δ*rffG*-associated cell envelope defects likely initiate the Rcs phosphorelay to subsequently

inhibit *flhDC* expression. To interrogate this model for the *rffG*-deficiency, we utilized the fact

that *flhDC* overexpression in wild-type *P. mirabilis* drives elongation of swarmer cells (53). The

resultant swarm colony forms a thin film without a concentric ring pattern, termed a

"hyperswarming" phenotype (14). We overexpressed *flhDC* in the WT and Δ*rffG* strain

backgrounds, resulting in WT + *flhDC* and Δ*rffG* + *flhDC*, respectively; swarm colonies of the

resultant strains both exhibited hyperswarming (Figure SF3A). We also observed these

populations by epifluorescence light microscopy using strains in which the *fliA*-venus

transcriptional reporter had been introduced. After six hours of growth on a swarm permissive

surface, both WT + *flhDC* and Δ*rffG* + *flhDC* populations were comprised of motile, elongated

cells with comparable fluorescence due to the *fliA* reporter (Figure 4A-B). We considered that

the swarming recovery in the *flhDC* overexpression strains might be due to restoration of the

HMW LPS-associated moieties. We analyzed the LPS fractions of Δ*rffG* + *flhDC* cells and

observed that the HMW LPS-associated moieties remained absent (Figure SF3B). Therefore,

over-expression of *flhDC* was not sufficient to restore production of this OM component (Figure

SF3B), which in itself indicates that this *rffG*-generated HMW OM component is not physically

required for swarmer cell elongation and motility. We further concluded that increased

expression of *flhDC* is sufficient to restore swarmer cell elongation, flagellar gene expression,

and motility to Δ*rffG* cells independently of OM structure.

These results confirmed that the inhibition of elongation in Δ*rffG* cells is due to a

signaling cascade. As RcsB acts upstream of *flhDC*, we hypothesized that deletion of *rcsB* would

also rescue swarmer cell elongation and motility, resulting in a phenotype equivalent to that of

the *flhDC* overexpression strains. However, direct comparisons of *flhDC* overexpression and

rcsB deletion are not readily apparent in the literature. We generated chromosomal deletions of

rcsB in the WT and Δ*rffG* strain backgrounds, resulting in Δ*rcsB* and Δ*rffG*Δ*rcsB*, respectively.

We confirmed that deletion of *rcsB* did not rescue the LPS-associated defects of Δ*rffG* (Figure

SF3B) and then tested the ability of these populations to engage in swarm motility. The Δ*rcsB*

populations formed hyperswarming colonies that covered a 10-cm surface in 24 hours (Figure

SF3C), which is consistent with previous reports (14). Surprisingly, swarm colonies of the

Δ*rffG*Δ*rcsB* population appeared mucoid with tight concentric swarm rings and expanded to less

than half the distance of the WT population (Figure SF3C), indicating that the Δ*rffG*Δ*rcsB* were

not fully rescued for swarm motility. To further characterize this swarming defect, we examined

Δ*rcsB* and Δ*rffG*Δ*rcsB* strains encoding the *fliA*-Venus transcriptional reporter by

epifluorescence light microscopy after six hours of growth on a swarm permissive surface. Cells

in the Δ*rcsB* populations were generally elongated and motile with increased fluorescence

expression, similar to cells of the WT + *flhDC* population (Figure 4C). Cells in the Δ*rffG*Δ*rcsB*

population, however, exhibited striking cell shape defects; cells were bloated, uneven in width,

formed spheres or tapering at the cell poles, or bulged at the mid-cell (Figure 4D). In addition,

several Δ*rffG*Δ*rcsB* cells were forked at the cell pole or branched at the mid-cell (Figure 4D).

The elongated cells in the Δ*rffG*Δ*rcsB* population also exhibited fluorescence, indicating that *fliA*

expression was restored, and cells engaged in swarm motility (Figure 4D). Altogether, loss of

rcsB was sufficient to partially restore swarmer cell elongation and motility to Δ*rffG* cells.

However, RcsB was also necessary for maintenance of proper cell shape in these envelope-

compromised cells.

Discussion

294   This research provides initial evidence that deletion of *rffG*, a predicted ECA

biosynthesis gene, inhibits *P. mirabilis* swarmer cell elongation. These results add to a growing

understanding of the importance of cell envelope composition to *P. mirabilis* swarming and

expands upon previous studies showing that OM structure directly regulates early stages of

swarmer cell development. Abrogation of O-antigen structure through deletion of the O-antigen

ligase (*waaL*) or chain length determinant (*wzz*) inhibits surface sensing, the earliest stage in the

swarmer cell cycle (22). The authors proposed that O-antigen mechanically senses the surface

and communicates this information via the Umo system (15). Here we propose that *P. mirabilis*

cells likely require additional cell envelope structures for swarmer cell elongation and possibly

surface sensing. Loss of *rffG* leads to an altered profile for LPS-linked moieties; cells lacked

uncharacterized HMW LPS-linked moieties and appeared to assemble a modified O-antigen

ladder. Based on the proposed function of the *rffG* gene product, we hypothesize that the HMW

LPS-linked moieties may be ECA or ECA-derived. However, efforts to confirm the presence of

LPS-linked ECA in *P. mirabilis* strain BB2000 have been unsuccessful to date, and further work

must be done to characterize the structural changes to the LPS. Regardless, this research supports

that the *rffG* gene is critical for the production of cell envelope components necessary for

swarming.

In addition to changes in OM structure, we also observed that *rffG*-deficient cells were

more susceptible to cell envelope stress. While *rffG* has been linked to ECA production, this

result suggests that deletion of *rffG* leads to pleiotropic effects impacting overall cell envelope

integrity. This is not surprising as perturbation of ECA or LPS biosynthesis genes result in the

accumulation of dead-end intermediates that broadly impact cell envelope integrity in *E. coli* (12,

13). Peptidoglycan, LPS, and ECA biosynthesis pathways share substrates, such as undecaprenyl

phosphate or lipid I (12, 13). Perturbation of the flux or sequestration of these shared substrates

across pathways broadly impact cell envelope integrity and can result in shape defects (12, 13).

Furthermore, previous work suggests that OM structure plays a crucial mechanical role in

resisting turgor pressure fluctuations associated with cell wall stressors, specifically beta-lactam

drugs (54). However, it remains unclear whether loss of the HMW LPS-linked moieties results in

compromised OM integrity and if such OM defects alone are sufficient to sensitize *rffG*-deficient

P. mirabilis cells to shape defects. Intriguingly, shape defects were not apparent in *rffG*-deficient

cells except for the subset of cells that undergo elongation on surfaces. It remains unclear why a

subpopulation of Δ*rffG* cells escape inhibition of elongation, or how factors leading to this

escape might contribute to cell shape. Additionally, loss of *rffG* resulted in broad changes to the

P. mirabilis transcriptome on surfaces. These changes included increased production of several

fimbrial gene clusters in concert with repression of flagellar and chemotaxis genes, along with

altered expression of several metabolism genes. Several defense-associated genes and stress-

associated transcription factors (*rcsA* and *rpoS*) were also up-regulated, suggesting the cells may

be activating stress-related pathways. We surmise that *rffG*-deficient cells enter into a

transcriptional state distinct from that of the swarm cycle; it is unclear whether this lifestyle

would persist for disruptions in other cell envelope biosynthesis pathways.

334   While several genetic pathways were perturbed, a central cell response through the Rcs

phosphorelay and *flhDC* appeared to be sufficient to restrict initiation of swarmer cell

development. Yet remarkably, we found that overexpression of *flhDC* and deletion of *rcsB* in

rffG-deficient cells did not result in the same phenotype; concurrent removal of *rcsB* and *rffG*

caused shape defects among swarming cells. These shape defects were not observed in Δ*rffG*

cells over-expressing *flhDC,* suggesting that neither expression of *flhDC* or elongation are

sufficient to induce shape defects. These observations support a novel role for Rcs in maintaining

the fidelity of the rod shape during swarmer cell elongation. As cells with intact cell envelopes

appear to have no requirement for *rcsB* to maintain shape, we surmise that a function for RcsB in

maintaining cell shape might be limited to cells with compromised envelopes. In fact, the Rcs

phosphorelay was recently proposed to act as a multifunctional regulator of swarmer cell

development through distinct modulation of motility and cell division regulators (27, 28). Our

results expand an understanding of the important and complex role that the Rcs phosphorelay has

in swarmer cell development and motility.

Remaining unclear is why constitutive expression of *flhDC* was sufficient to restore

swarmer cell elongation to Δ*rffG* cells while maintaining cell shape and polarity. Based on the

fact that the Rcs phosphorelay responds to cell stress, we predict that RcsB may play a role in

maintaining cell envelope integrity through unknown mechanisms in these *flhDC*-overexpression

cells. If so, constitutive expression of *flhDC* under a non-native promoter likely uncoupled the

flhDC-repressive and cell envelope protective activities of RcsB. Thus, Δ*rffG* + *flhDC* cells may

represent an artificial system in which cells are driven to elongate while maintaining the cell

envelope protective effects of Rcs activation (Figure 5). As such, we deduce that the cell shape

protective effects of RcsB occur upstream of *flhDC.* Thus, this work reveals a previously

underappreciated *flhDC*-independent pathway linking RcsB to swarmer cell elongation.

Others have also proposed that Rcs plays a conditional role in cell shape maintenance in

gram-negative bacteria. L-form *E. coli* cells require the Rcs stress response to recover a rod

shape; cells lacking this response lyse (29). The authors of that study proposed that Rcs may

function to maintain cell shape in conditions in which cells may lose cell wall through exposure

to lysozyme or cationic antimicrobial peptides, including several niches within a human host

(29). In addition to overall bloating and irregularity of width, cells lacking both *rffG* and *rcsB*

cells exhibited perturbation of cell pole structure and growth from the mid-cell. To our

knowledge, we are the first to describe such defects in *P. mirabilis.* However, similar phenotypes

have been described in *E. coli* and *Agrobacterium tumerfaciens.* In those organisms, cell polarity

defects appeared to arise from the formation of patches of inert peptidoglycan and mislocalized

division planes (18-21, 55). Further study is needed to mechanistically understand which aspects

of the RcsB regulon are specific for cell shape maintenance.

Several questions also remain regarding the mechanism of activation and the downstream

activity of RcsB in *rffG*-deficient cells. First, Bode et al recently demonstrated that MrpJ also

acts as a regulator toggling cells between swarm motility (MrpJ-repressed) and non-motile

adherence (MrpJ-induced) (51); RcsB positively regulates MrpJ (27, 28). However, the genes

differentially regulated in Δ*rffG* (Table S1, S2) neither fully overlap with the RcsB regulon (27,

28) or the MrpJ regulon (51). Clearly additional pathways are affected in the *rffG*-deficient cells;

one such candidate is the RpoE regulon (16). And pathways connecting RcsB and MrpJ to

swarmer cell elongation and *flhDC*-dependent motility need to be more fully characterized.

Second, perturbation of OM structure appears to be communicated through both RcsF-dependent

and independent pathways in *P. mirabilis* (15). It remains unclear whether cell envelope stress of

the *rffG*-deficient cells is communicated through the OM-localized RcsF, through the

upregulation of RcsA, or through an uncharacterized additional pathway. Also unknown is

whether the *rffG*-dependent HMW LPS-linked moieties act through the Umo system, as was

previously shown for O-antigen (15). Finally, the DNA-binding activity of RcsB has been shown

to be modulated by both phosphorylation and association with auxiliary transcription factors in

E. coli (56). How RcsB activity is modulated downstream of phosphorylation remains unclear in

P. mirabilis.

Taken together with recent research on the Rcs regulon in *P. mirabilis* (27, 28), we

propose that the Rcs phosphorelay functions as a developmental checkpoint for the swarm cell

cycle (Figure 5). Under swarm-friendly conditions in the presence of wild-type cell envelope

structure, Rcs would be inactive thereby allowing the swarm cell cycle to progress. By contrast,

the absence or alteration of the cell envelope structure likely activates the Rcs phosphorelay,

which in turn a) activates pathways protective against cell shape defects, b) activates cell

division pathways, and c) inhibits expression of *flhDC*. Based on the transcriptional profile of

Δ*rffG* cells and other cells activating the Rcs phosphorelay (27, 28), we predict that the activity

of Rcs culminates in the adaptation of an adherent or biofilm-promoting lifestyle that may

provide protection against external stressors. We propose that the HMW LPS-linked moieties,

which are potentially ECA, might provide one important signal that communicates cell integrity

to the Rcs system. As many species within the Enterobacteriaceae family express both ECA and

the Rcs phosphorelay, ECA and Rcs might serve as broad-acting regulators of bacterial cell

development and motility.

Experimental Procedures

<u>Growth conditions</u>

Liquid cultures were grown in LB-Lennox broth. Colonies were grown in 0.3 % LB agar for

swimming motility assays, on LSW- agar (57) for plating non-motile colonies, and on CM55

blood agar base (Oxoid, Hampshire, UK) for swarming. Antibiotics for selection were used

throughout all assays as following: 15 µg/mL tetracycline, 25 µg/mL streptomycin, and 35

µg/mL kanamycin. For swarm assays, overnight cultures were normalized to OD_{600} 1.0, and 1 µl

of culture was inoculated with a needle onto swarm-permissive CM55 blood agar base plates

containing kanamycin, 40 µg/mL Congo Red, and 20 µg/mL Coomassie Blue. Plates were

incubated at 37 °C. For swim assays, an inoculation needle was used to inoculate overnight

cultures into 0.3% LB containing kanamycin. Plates were incubated at 37 °C for 16 hours.

Images were taken with a Canon EOS 60D camera.

Strain construction

Strain construction was performed as described previously (58). For construction of the Δ*rcsB*

strain, a gBlock (Integrated DNA Technologies, Coralville, IA) containing the 452 base-pairs

(bp) upstream and downstream of *rcsB* (*P. mirabilis* BB2000, accession number CP004022:nt

1972701…1973153 and 1973809…1974261) was generated and introduced to pKNG101 at SpeI

and XmaI sites using Slice cloning (59). Similarly, for construction of the Δ*rffG* strain, a gBlock

containing a chloramphenicol resistance cassette (amplified from pBAD33 (60)) flanked by 1000

bp upstream of *rffG* and downstream of *rffG* (*P. mirabilis* BB2000, accession number

CP004022:nt 3635400…3636399 and 3637473…3638473) was introduced to pKNG101 (58) at

the same sites. Expression plasmids were introduced into *P. mirabilis* via *E. coli* SM10lpir as

previously described (57). Resultant strains were confirmed by Polymerase Chain Reaction

(PCR) of the targeted region. The Δ*rffG* strain was confirmed through whole genome sequencing

as described in (61).

For construction of *fliA* reporter strain, a gBlock encoding the last 500 bp *fliA* (*P. mirabilis*

BB2000, accession number CP004022:nt 1856328…1856828), RBS (aggagg), a modified

variant of Venus fluorescent protein (a gift from Drs. Enrique Balleza and Phillipe Cluzel), and

500bp downstream *fliA* (*P. mirabilis* BB2000, accession number CP004022:nt

1855828…1856328) was inserted into pKNG101 (58) at the ApaI and XbaI sites. Expression

plasmids were introduced into *P. mirabilis* via *E. coli* SM10lpir as previously described (57).

Resultant strains were confirmed by PCR of the targeted region and sequenced.

For construction of the *rffG* expression strains, the nucleotide sequence for *rffG* was PCR

amplified from the *P. mirabilis* chromosome using oKL273 and oKL274 and inserted into

expression vector pBBR1-NheI (62) using AgeI and NheI restriction enzyme sites. For the *flhDC*

expression strains, the nucleotide sequences *flhDC* (amplified using oKL277 and oKL278) was

similarly inserted into pBBR1-NheI. A gBlock containing the *lac* promoter (sequence:

cacggtcacactgcttccggtagtcaataaaccggtgcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacacttt

atgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgaaggatcctaggag

gtactagtatg) was introduced upstream of the coding region using Slice (59). Plasmids were

confirmed by Sanger Sequencing (Genewiz, South Plainfield, NJ). Expression plasmids were

introduced into *P. mirabilis* via *E. coli* S17lpir as previously described (57).

<u>LPS extraction and analysis</u>

Cells were grown on overnight at 37°C on swarm-permissive agar and harvested with LB. LPS

from cells was extracted using an LPS Extraction Kit according to manufacturer's instructions

(iNtRON Biotechnology Inc, Sangdaewon Seongnam, Gyeonggi, Korea). Extracts were

resuspended in 10mM Tris, pH 8.0 buffer and run on a 12% SDS-PAGE gel. Gels were stained

with a modified silver stain protocol (36).

<u>Bile salts resistance assays</u>

Cultures were grown overnight, normalized to OD 1.0, and serial diluted 10-fold. 1 µL spots of

10^{-1} to 10^{-8} dilutions of each strain (in technical triplicate) were inoculated onto LSW- plates

containing bile salts (filter sterile, added to medium post-autoclaving). Plates were incubated

overnight at 37°C.

<u>Carbenicillin sensitivity assay</u>

Cells were harvested from swarm permissive medium using LB broth and spread onto 1 mm agar

pads containing swarm permissive medium with 10 µg/mL carbenicillin (Corning, Corning,

NY). Cells were imaged after 1, 2, or 4 hours on surface. Three biological replicates were

analyzed.

<u>Microscopy</u>

Microscopy was performed as previously described (61). Briefly, swarm-permissive agar pads,

supplemented with 35 µg/mL kanamycin for plasmid retention, were inoculated from overnight

stationary cultures and incubated at 37°C in a modified humidity chamber. Pads were imaged

using a Leica DM5500B (Leica Microsystems, Buffalo Grove, IL) and a CoolSnap HQ2 cooled

CCD camera (Photometrics, Tuscon, AZ). MetaMorph version 7.8.0.0 (Molecular Devices,

Sunnyvale, CA) was used for image acquisition. Images were analyzed using FIJI (63) (National

Institutes of Health, USA); where indicated, images were subjected to background subtraction

(equally across entire image). Where indicated, cells were stained with 25 µM TMA-DPH

(Invitrogen, Carlsbad, CA), (max excitation 355 nm; max emission 430 nm) imaged in the DAPI

channel using an A4 filter cube (excitation 360/40 nm; emission 470/40 nm) (Leica

Microsystems, Buffalo Grove, IL). Venus (max excitation 515 nm; max emission 528 nm) was

visualized in the GFP channel using a GFP ET filter cube (excitation 470/40 nm; emission

525/50 nm) (Leica Microsystems, Buffalo Grove, IL). Fluorescence intensity and exposure times

for each fluorescence channel were equivalent across all fluorescence microscopy experiments.

Fluorescence due to Venus was not quantified and was visible due to the overlapping excitation

and emission spectra with the GFP ET filter cube.

<u>Transcriptional analysis</u>

Strains were grown on swarm-permissive agar plates at 37 ˚C. For WT samples, colonies were

inoculated on swarm-permissive agar and incubated overnight for swarm development. The

presence of short, non-motile cells in consolidation phase was confirmed by light microscopy.

Cells from the swarm edge were then harvested by scraping with a plastic loop into 1 ml of RNA

Protect solution (Qiagen, Venlo, Netherlands). The Δ*rffG* samples were harvested after overnight

incubation on swarm-permissive agar by scraping whole colonies into 1 ml RNA Protect

solution. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Venlo, Netherlands)

according to the manufacturer's instructions. RNA purity was measured using an Agilent 2200

Tapestation (Agilent, Santa Clara, CA). To enrich mRNA, rRNA was digested using terminator

5' phosphate dependent exonuclease (Illumina, San Diego, CA) according to the manufacturer's

instructions and purified by phenol-chloroform extraction (64). cDNA libraries were prepared

from mRNA-enriched RNA samples using an NEBNext Ultra RNA library prep kit (New

England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Libraries were

sequenced on an Illumina NextSeq 2500 instrument with 250-basepair single-end reads.

Sequences were matched to the BB2000 reference genome (accession number CP004022) using

Tophat 2 (65). Differential expression data were generated using the Cufflinks RNA-Seq

analysis suite (66) run on the Harvard Odyssey cluster, courtesy of the Research Computing

Group. Data were analyzed using the CummeRbund package for R and Microsoft Excel (66).

Gene functions were ascribed based on COG annotations (67). Bioinformatics information was

derived from KEGG (68). The data in this paper represent the combined analysis of two

independent biological repeats.

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Conflicts of Interest

519   The authors declare no conflicts of interest.

Author Contributions

522   KL and KAG conceived and coordinated the study and wrote the paper. KL and KAG designed

523   all experiments; MJT performed RNA-Seq (Figure 3; Table 1, 2, S1, S2). KL all other

524   experiments. All authors edited the paper and approved the final version.

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	Figure Legends

Figure 1: Loss of *rffG* affects OM structures and cell envelope integrity

A. Silver-stained LPS extracted surface-grown WT, Δ*rffG*, and *rffG*-complemented populations.

LPS-associated moieties are labeled on the left.

B. Cultures of the WT, Δ*rffG,* and *rffG*-complemented strains were normalized based on OD_{600},

and 10-fold serial dilutions were spotted on indicated % bile salt media. Images are

representative, n = 3.

C. WT and Δ*rffG* strains were spread onto swarm-permissive agar pads containing 0 or 10

µg/mL Cb. Shown are cells after one, two, or four hours of incubation on a surface. Images are

representative; n =3. Scale bars = 10 µm.

Figure 2: Loss of *rffG* inhibits swarmer cell elongation and swarm motility

A. WT and Δ*rffG* strains were grown on swarm-permissive medium, on which cells engage in

the swarm cycle (see 2B) and remain along the top of the surface.

B. Cartoon depictions of the morphological state of cells grown on swarm permissive solid

medium. On surfaces, cells elongate up to 40-fold before engaging in motility and dividing into

short (1-2 µm) rigid rods.

C. Phase contrast and epifluorescence microscopy of WT, Δ*rffG,* or *rffG*-complemented

populations after two, four, and six hours on a swarm-permissive surface. Strains carried either

an empty vector or expression vector to allow growth on selective medium. Venus fluorescence

corresponds to *fliA* reporter expression and is shown for the six-hour time point. Rolling ball

background subtraction was performed using FIJI (63). Magenta arrow highlights an elongating

cell in the Δ*rffG* population that is bulging. Frames from a time-lapse of such cells bulging are in

Figure SF2. Scale bars = 10 μm.

Figure 3: The expression profile of Δ*rffG* cells is distinct from that of WT on surfaces

A. Graphical summary of genes differentially regulated in Δ*rffG* versus short, non-motile WT

cells grown on a swarm-permissive surface. The x-axis corresponds to each gene on the *P.*

mirabilis strain BB2000 chromosome. The y-axis corresponds to a $\log_2$ fold difference in gene

expression. All data are shown. Red circles indicate genes that are also regulated by RcsB as

determined in (27, 28). The most highly altered gene (*mrpA* at ~ 200-fold) is labeled.

B. Summary of the COG functional categories (67) for the significant differentially regulated

genes.

Figure 4: RcsB and FlhDC rescue swarm elongation of *rffG*-deficient cells; RcsB is

additionally essential for maintaining cell shape

Epifluorescence microscopy of (A) WT + *flhDC*, (B) Δ*rffG* + *flhDC*, (C) Δ*rcsB*, and (D)

Δ*rffG*Δ*rcsB* populations; all strains carry the chromosomal *fliA* reporter. Images were taken once

swarm motility initiated (~4-6 hours on surface). Rolling ball background subtraction on *fliA*

reporter images was conducted using FIJI (63). Magenta arrows indicate a cell exhibiting shape

and polarity defects. Left, phase contrast; middle, membrane stain; right, Venus expression.

Scale bars = 10 μm.

Figure 5: Rcs activation inhibits cell elongation while maintaining cell envelope integrity.

Activation of the Rcs phosphorelay results in activation of adherence-promoting genes when the

membrane is compromised. We hypothesize that artificial, constitutive expression of *flhDC*

under the *lac* promoter can uncouple Rcs repression of *flhDC* from activation these pathways,

thereby allowing full rescue of Δ*rffG* swarmer cell elongation through constitutive expression of

flhDC.

Strain Table

Name	Description	Source
WT	Wild type strain	(57)
Δ*rffG*	BB2000Δ*rffG::CmR*	This study
Δ*rcsB*	BB2000Δ*rcsB*	This study
Δ*rffG* complemented	BB2000Δ*rffG*/pBBR1-P(*lac*)-*rffG*	This study
WT + *flhDC*	BB2000/pBBR1-P(*lac*)-*flhDC*	This study
Δ*rffG* + *flhDC*	BB2000Δ*rffG*/pBBR1-P(*lac*)-*flhDC*	This study
Δ*rffG*Δ*rcsB*	BB2000Δ*rcsB*Δ*rffG::CmR*	This study
WT::*fliA, Venus*	BB2000 with ribosomal binding site (RBS) and Venus inserted immediately downstream of *fliA* stop codon	This study; Venus construct from P. Cluzel
WT::*fliA, Venus* + *flhDC*	WT::*fliA, Venus*/pBBR1-P(*lac*)-*flhDC*	This study
Δ*rffG::fliA, Venus*	Δ*rffG* with (RBS) and Venus inserted immediately downstream of *fliA* stop codon	This study

Δ*rffG*::*fliA, Venus* + *flhDC*	Δ*rffG*::*fliA, Venus*/pBBR1-P(*lac*)-*flhDC*	This study
Δ*rcsB*::*fliA, Venus*	Δ*rcsB* with (RBS) and Venus inserted immediately downstream of *fliA* stop codon	This study
Δ*rffG*Δ*rcsB*::*fliA, Venus*	Δ*rffG*Δ*rcsB* with (RBS) and Venus inserted immediately downstream of *fliA* stop codon	This study
WT + empty vector	BB2000/pBBR1-NheI	This study
Δ*rffG* + empty vector	BB2000Δ*rffG*::*CmR*/pBBR1-NheI	This study

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769   **Plasmid Table**

Name	Descriptions	Source
empty vector	pBBR1-NheI	(62)
+ *rffG*	pBBR1-P(lac)-r*ffG*	This study
+ *flhDC*	pBBR1-Pr(lac)-*flhDC*	This study

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773   **Table 1: Top 20 genes up-regulated in Δ*rffG* relative to WT**

Transcript	Product	Fold Change	Rcs Regulon?*
mrpA	major mannose-resistant/Proteus-like fimbrial protein	200.27	Yes
caiF	DNA-binding transcriptional activator CaiF	63.60	
pmpA	fimbrial subunit	55.46	
BB2000_1924	transcriptional regulator	45.63	
BB2000_1499	fimbrial subunit	44.85	
BB2000_3017	fimbrial operon regulator	44.59	
zntB	zinc transporter	42.83	
mrpG	fimbrial subunit	42.72	
BB2000_0667	hypothetical protein		
ompW	outer membrane protein W	33.70	
BB2000_2725	TetR-family transcriptional regulator	32.64	
dcuB	anaerobic C4-dicarboxylate transporter	29.58	
BB2000_0531	sigma 54 modulation protein	25.53	

BB2000_0331	heat shock protein HtpX	22.81	
BB2000_0299	hypothetical protein	22.47	
BB2000_0395	hypothetical protein	21.42	Yes
BB2000_0229	hypothetical protein	21.19	
BB2000_1497	fimbrial chaperone protein	20.97	
mrpJ	fimbrial operon regulator	19.08	
dmsA	dimethyl sulfoxide reductase chain A	18.99	Yes

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776   * Based on the published RcsB regulon in *P. mirabilis* (27, 28)

779 **Table 2: Top 20 genes down-regulated in ΔrffG relative to WT**

Transcript	Product	Fold Change	Rcs Regulon?*
cspB	cold shock protein	0.01	
flgN	flagella synthesis protein	0.03	
BB2000_1016, BB2000_1017	cold shock protein, heat shock protein	0.04	
flgM	anti-sigma28 factor FlgM	0.04	
BB2000_3499	lipoprotein	0.05	Yes
fliE	flagellar hook-basal body complex protein	0.05	
BB2000_1271	putative MFS-type transporter YdeE	0.07	
fliA	flagellar biosynthesis sigma factor	0.08	Yes
BB2000_1466	hypothetical protein		Yes
holD	DNA polymerase III subunit psi	0.10	Yes
intB	prophage integrase	0.11	
fliM, fliN	flagellar motor switch protein FliM, flagellar motor switch protein FliN	0.11	Yes
BB2000_0342	transcriptional regulator	0.11	Yes

BB2000_0996, umoD	hypothetical protein, exported protein (upregulator of flagellar master operon)	0.12	Yes
rpsO	30S ribosomal protein S15	0.12	
ccm	membrane protein (Ccm1 protein)	0.12	Yes
rplI	50S ribosomal protein L9	0.12	
fliZ	flagella biosynthesis protein FliZ	0.12	Yes
BB2000_2557	phospholipid-binding protein	0.13	Yes
BB2000_2289, fruK	hypothetical protein, 1-phosphofructokinase	0.13	

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782   * Based on the published RcsB regulon in *P. mirabilis* (27, 28)

Legend:
Figure 1: Loss of *rffG* affects OM structures and cell envelope integrity
A. Silver-stained LPS extracted surface-grown WT, Δ*rffG*, and *rffG*-complemented populations. LPS-associated moieties are labeled on the left.
B. Cultures of the WT, Δ*rffG*, and *rffG*-complemented strains were normalized based on OD$_{600}$, and 10-fold serial dilutions were spotted on non-swarm permissive medium with indicated % bile salt media. Images are representative, n = 3.
C. WT and Δ*rffG* strains were spread onto swarm-permissive agar pads containing 0 or 10 μg/mL Cb. Shown are cells after one, two, or four hours of incubation on a surface. Images are representative, n =3. Scale bars = 10 μm.

Legend:
Figure 2: Loss of *rffG* inhibits swarmer cell elongation and swarm motility
A. WT and Δ*rffG* strains were grown on swarm-permissive medium, on which cells engage in the swarm cycle (see 2C) and remain along the top of the surface.
B. Cartoon depictions of the morphological state of cells grown on swarm permissive media. On surfaces, cells elongate up to 40-fold before engaging in motility and dividing into short (1-2 μm) rigid rods.
C. Phase contrast and epifluorescence microscopy of WT, Δ*rffG*, or *rffG*-complemented populations after two, four, and six hours on a swarm-permissive surface. Strains carried either an empty vector or expression vector to allow growth on selective medium. Venus fluorescence corresponds to *fliA* reporter expression and is shown for the six-hour time point. Rolling ball background subtraction was performed using FIJI (61). Magenta arrow highlights an elongating cell in the Δ*rffG* population that is bulging. Frames from a time-lapse of such cells bulging are in Figure SF2. Scale bars = 10 μm.

Legend:

Figure 3: The expression profile of ΔrffG cells is distinct from that of WT on surfaces

A. Graphical summary of genes differentially regulated in Δ*rffG* versus short, non-motile WT cells grown on a swarm-permissive surface. The x-axis corresponds to each gene on the *P. mirabilis* strain BB2000 chromosome. The y-axis corresponds to a log2-fold difference in gene expression. All data are shown. Red circles indicate genes that are also regulated by RcsB as determined in (30). The most highly altered gene (*mrpA* at ~ 200-fold) is labeled.

B. Summary of the COG functional categories for the significant differentially regulated genes (65).

| Phase | Membrane dye | *fliA* reporter |

A

B

C

D

Legend:
Figure 4: RcsB and FlhDC rescue swarm elongation of *rffG*-deficient cells; RcsB is additionally essential for maintaining cell shape

Epifluorescence microscopy of (A) WT + *flhDC*, (B) Δ*rffG* + *flhDC*, (C) Δ*rcsB*, and (D) Δ*rffG*Δ*rcsB* populations; all strains carry the chromosomal *fliA* reporter. Images were taken once swarm motility initiated (~4-6 hours on surface). Rolling ball background subtraction on fliA reporter images was conducted using FIJI (61). Left, phase contrast; middle, membrane stain; right, Venus expression. Scale bars = 10 µm.

Legend:
Figure 5: Rcs activation inhibits cell elongation while maintaining cell envelope integrity.
We hypothesize that activation of the Rcs phosphorelay results in activation of cell envelope-protective pathways during times of stress. We posit that artificial expression of *flhDC* under the lac promoter uncouples Rcs repression of *flhDC* from activation these protective pathways, thereby allowing full rescue of Δ*rffG* swarmer cell elongation through constitutive expression of *flhDC*.