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Investigating Flagella-Driven Motility in Escherichia coli by Applying Three Established Techniques in a Series

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Cover letter

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Dear Editor,

This manuscript is a response to an invitation from Jaydev Upponi to submit to your journal (December 5th, 2019) stemming from our recently published paper in the Journal of Bacteriology "*Under elevated c-di-GMP in E. coli, YcgR alters flagellar motor bias and speed sequentially, with additional negative control of the flagellar regulon via the adaptor protein RssB"* DOI: 10.1128/JB.00578-19. Therein, we deploy a number of techniques to uncover the nuances of motility inhibition in response to the second messenger c-di-GMP. In this manuscript, we detail some of the techniques crucial to that work, and combine them in series with other methods, to exploit motility as a selection tool to identify components/pathways contributing to swimming and swarming motility, and then characterize them.

Though the protocols we describe relate to *E. coli*, they can be readily adapted for application in other species, and offer elegant and practical ways to gain insight into bacterial motility. Correspondence with your team implied that if accepted, publication might be feasible by May, which would be advantageous to us.

We hope you will find this work of interest to your journal and of assistance to your readers.

Yours faithfully,

Jonathan Partridge Ph.D.

TITLE:

Investigating Flagella-Driven Motility in *Escherichia coli* by Applying Three Established Techniques in a Series

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bacteria, chemotaxis, *Escherichia coli*, flagella, rotary motor, motility, swimming, swarming, surface sensing, surface motility

SUMMARY:

Many bacteria use flagella-driven motility to navigate their environment and colonize favorable surroundings both individually and as a collective. Demonstrated here is the use of three established methods that exploit motility as a selection tool to identify components/pathways contributing to swimming and swarming motility.

ABSTRACT:

Motility is crucial to the survival and success of many bacterial species. Many methodologies exist to exploit motility to understand signaling pathways, to elucidate the function and assembly of flagellar parts, and to examine and understand patterns of movement. Here we demonstrate a combination of three of these methodologies. Motility in soft agar is the oldest, offering a strong selection for isolating gain-of-function suppressor mutations in motility-impaired strains, where motility is restored through a second mutation. The cell-tethering technique, first employed to demonstrate the rotary nature of the flagellar motor, can be used to assess the impact of signaling effectors on the motor speed and its ability to switch rotational direction. The "border-crossing" assay is more recent, where swimming bacteria can be primed to transition into moving collectively as a swarm. In combination, these protocols represent a systematic and powerful approach to identifying components of the motility machinery, and to characterizing their role in different facets of swimming and swarming. They can be easily adapted to study motility in other bacterial species.

INTRODUCTION:

Bacteria employ many appendages for movement and dispersal in their ecological niches[1]. Flagella-driven motility is the fastest of these, promoting the colonization of favorable niches in response to environmental signals, and contributing significantly to the pathogenic ability of

some species[2,3]. Flagellated bacteria can swim individually in bulk liquid, or swarm as a collective over a semi-solid surface[4]. Extracellular flagella attach to and are driven by rotary motors embedded in the membrane, which harness the power of ion gradients to generate torque that causes rotation[1,2,4-8]. In *E. coli*, whose motors run at a constant torque[9], the motor output can be categorized in terms of rotational speed and switching of the rotor between counter-clockwise (CCW) and clockwise (CW) directions. CCW rotation promotes formation of a coherent flagellar bundle that propels the cell forward (run), while a transient switch in rotational direction (CW) causes the bundle to disassemble either partially or fully[10], and the cell to reorient its swimming direction (tumble). *E. coli* typically run for a second and tumble for a tenth of a second. Switching frequency of the rotor or 'tumble bias' is controlled by the chemotaxis signaling system, wherein transmembrane chemoreceptors detect external chemical signals and transmit them via phosphorelay to the flagellar motor to extend runs in response to attractants, or suppress them in response to toxic chemicals[11,12]. Swimming motility is assayed in 0.3% soft agar.

During swarming, bacteria navigate on a semi-solid surface as a dense collective, where packs of bacteria stream in a continuous swirling motion[2,13-15]. *E. coli* swarms exhibit altered chemosensory physiology (lower tumble bias), higher speeds, and higher tolerance to antimicrobials over cells swimming in bulk liquid[16,17]. Swarmers vary in their deployment of a plethora of strategies that aid movement, including surfactant production, hyperflagellation, and cell elongation[2]. Swarming offers bacteria a competitive advantage in both ecological and clinical settings[18-20]. There are two categories of swarming bacteria: temperate swarmers, which can swarm only on media solidified with 0.5-0.8% agar, and robust swarmers, which can navigate across higher agar concentrations[21].

A variety of assays exist to interrogate swimming motility and its regulation. When impaired by mutations or environmental conditions, motility itself offers a strong selection for identifying gain-of-function suppressor mutations. These suppressors can be genuine revertants of the original mutation, or pseudo-revertants, where a second mutation restores functionality. Such mutants can be identified by whole genome sequencing (WGS). An alternative to unbiased suppressor selection is a biased targeted mutagenesis strategy (e.g., PCR mutagenesis). These methodologies often shed light on the function or environmental regulation of the motility apparatus. If the goal is to study motor function, then the restoration of wild-type motility as measured in soft agar may not necessarily indicate restoration of wild-type motor output. The cell-tethering assay, in which cells are attached to a glass surface by a single flagellum, and rotation of the cell body is subsequently monitored, can be the initial assay of choice for assessing motor behavior. Although more sophisticated methodologies are now available to monitor motor properties, the required high-speed camera set-up and application of software packages for motion analysis limit their widespread use[22-25]. The cell-tethering assay requires only that the flagella be sheared, allowing attachment of the short filaments to a glass slide, followed by videotaping the rotation of the cell body. Although the recorded motor speeds are low in this assay because of the high load the cell body exerts on the flagellum, this assay has nonetheless contributed to valuable insights into chemotactic responses[26-29], and remains a valid investigative tool as discussed below.

Swarming motility poses a different set of challenges to researchers. Selection of gain-of-function suppressors only works in swarmers that produce copious surfactants and swarm readily[13]. Surfactant non-producers such as *E. coli* are fastidious with respect to the choice of agar, media composition and humidity of the environment[2,13,14,21]. Once swarming conditions are established, the border-crossing assay[17] is a useful methodology to interrogate the ability of a swarm to navigate new/harsh conditions. Through the protocols presented below, we describe relate to *E. coli*, they can be readily adapted for application in other species.

PROTOCOLS:

1. Isolation of suppressor mutants in motility-deficient strains

NOTE: Use this method as a broad 'catch-all' to identify the general nature of the motility defect.

1.1. Soft-agar plate preparation

NOTE: Soft-agar, also referred to as motility- or swim-agar, is a low percentage agar (~0.2-0.35% w/v), long used to assay chemotaxis[31,32].

1.1.1. Add 3 g of bacto-agar (0.3% w/v) and 20 g of LB to a 2 L round bottom flask. Add 1 L of ddH_2O (double-distilled water) to the flask and evenly mix the suspension using a stir rod and magnetic stirring plate.

1.1.2. Autoclave for 20 min at 121 °C.

1.1.3. Allow to cool with gentle agitation using the rod/plate as above. When the temperature reaches approximately 50 °C, pour 25 mL into sterile Petri dishes (100 mm x 15 mm), and allow the molten agar to set with lid in place for at least 1 h, for use within 16 h.

1.2. Culture preparation, inoculation, and isolation of suppressor mutants

NOTE: *E. coli* inoculated in the center of nutrient rich media solidified with soft agar consume nutrients locally, creating a nutrient gradient that they follow. As they move outward, defined 'rings' appear (**Figure 1A**), which are related to specific chemoattractants the bacteria respond to. Defects in either the chemotaxis system or structural components of the flagella motor can compromise performance in this assay. Often, mutants with a motility advantage arise during screening, and can be seen emerging from single or from multiple points along the periphery of the ring, from where they 'flare' out (**Figure 1C**). One will notice that the outermost edge of the swimming front contrasts readily against the uncolonized virgin soft agar.

1.2.1 Grow overnight cultures of the desired motility-deficient strain in 5 mL of Lennox Broth (LB; 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, **Table of Materials**) at 30 °C with horizontal shaking (220 r.p.m.). Sub-culture (1:100 dilution) in fresh LB, growing under the same conditions to exponential phase (OD_{600} of 0.6).

1.2.2. Inoculate 6 µL of the culture into the center of a soft-agar plate (1.1) using a pipette, pushing the loaded sterile tip into the agar to gently expel the contents. Transfer to 30 °C and incubate (**Figure 1B**), until motility 'flares' are evident, emanating from the inoculation point or the periphery of the motility rings, typically in 24-36 h (**Figure 1C**).

NOTE: In motility assays, inoculate a wild-type strain alongside the mutant isolates for comparison. This wild-type strain will show the characteristic concentric chemotactic rings (**Figure 1A**) and fill the plate within 8-10 h.

1.2.3. Use a sterile wire-loop to lift the cells from the 'flare' region and streak to purify single colonies onto an LB hard agar plate (LB prepared as above, solidified with 15 g/L bacto-agar).

1.2.4. Pick single colonies from the streak plate using a sterile wire loop and re-purify by streaking for single colonies to ensure isolation of a 'pure' colony isolate.

1.3. Confirmation, and characterization of suppressor mutants

1.3.1. Confirm that the isolated suppressor mutant(s) have restored motility. Prepare soft-agar plates (1.1), and cultures for strains of interest (as in 1.2.1), including wild-type and the starting 'motility-deficient' strain for comparison.

1.3.2. Inoculate the plates (as in 1.2.2) and incubate at 30 °C for 8-10 h.

1.3.3. Record the diameter of the outermost ring (edge of the circle) and compare to establish which of the isolates have substantially restored motility.

NOTE: It is recommended that plates be photographed throughout the time-course of the experiment. For best results, use a "bucket of light" device[30], where a digital camera is mounted above a light source for better illumination to measure the diameter of the swim colony and distinguish it from uncolonized agar.

1.3.4. Subject verified isolates to WGS as required, allowing for sufficient 'sequence coverage' to positively identify the mutations that restored wild-type function.

2. Quantifying flagella motor behavior via cell tethering

NOTE: Use this method when normal run-tumble behavior (chemotaxis) appears to be compromised.

2.1. Culture preparation and flagella shearing

2.1.1. Prepare an exponential phase culture of the strain of interest as described in step 1.2.1.

2.1.2. Pellet 10 mL of cells by centrifugation at 2,000 *x g* for 3 min before resuspending in 10 mL of filter-sterilized Motility Buffer (MB; 10 mM potassium phosphate buffer [0.0935 M K_2HPO_4, 0.0065 M KH_2PO_4, pH 7.0], 0.1 mM EDTA [pH 7.0], 10 mM NaCl, 75 mM KCl).

NOTE: MB supports motility, but does not support bacterial growth

2.1.3 Repeat step 2.1.2 two more times before resuspending the final pellet in 1 mL of MB.

2.1.4. Transfer the cell suspension into a 1 mL syringe and attach a 23G needle to the end. Assemble an identical syringe/needle apparatus and attach the two together via 6 inches of polyethylene tubing (inner diameter of 0.58 mm) tightly sheathed over each needle tip.

2.1.5. Shear the flagella (they are fragile and break easily) by gently passing the cell suspension back and forth from one syringe to the other 50x, with 1 min pauses between every 10 passes.

2.1.6. Centrifuge the sheared cells at 2,000 *x g* for 3 min and resuspend in a final volume of 500 µL of MB.

2.2. Slide preparation and cell tethering

2.2.1. Prepare a cell fixation chamber by stacking an 18 mm x 18 mm coverslip over a 3 inch x 1 inch x 1 mm glass microscope slide, separated by double-sided tape (**Figure S1**).

2.2.2. Flush the chamber with 0.01% (w/v) poly-lysine solution by applying to the top of the chamber Tilt the bottom edge (the coverslip flush with the microscope slide) onto a task wiper (tissue paper) to help draw solution through the chamber (**Figure S1**). Then incubate at room temperature for 10 min.

2.2.3. Wash the chamber three times with 40 µL of MB using as described in step 2.2.2

2.2.4. Add 40 µL of the sheared cell suspension (prepared above) to the top of the chamber and incubate at room temperature for 10 min to allow cells to attach to the coverslip.

2.2.5. Gently flush the chamber with 40 µL of MB as in 2.2.3 to remove unattached cells.

2.3. Cell rotation recording and quantification

2.3.1. Transfer the microscope slide loaded with tethered cells to the microscope stage.

2.3.2. Using phase-contrast microscopy and a 100x objective, scan the population for cells that are fixed in place, and rotating on a single axis, i.e., smooth rotations on a fixed point rather than presenting at an angle wherein the cell moves in and out of focus (**Video 1**).

2.3.3. Use a commercial microscope and associated camera. Open the associated software,

ensure cells of interest are in focus and click **video acquisition** to record the cell rotation for one minute (at 10 frames per second or higher).

2.3.4. From video playback, quantify the number of complete rotations per minute and the number of times the cell changes direction (switching frequency).

NOTE: Rotational speeds and switching frequency may be too fast to gauge by eye so it is recommended to use video software that offers slow/fine playback, or adopts an automated software system to quantify rotational patterns[33]. An alternative would be to increase the viscosity of the MB using methyl cellulose (or similar agent) to help slow and resolve the rotation of faster bacteria or compensate when cameras with low framerates are in use.

2.3.5. Repeat step 2.3.2 with biological replicates to compile a representation of the population of interest.

3. Preparation of swarms in a border-crossing assay

NOTE: Use this method for assessing the impact of a mutation or condition on group motility. Swarm-agar refers to agar where the percentage is typically higher than that of soft-agar. In soft agar (0.3 %), cells swim individually inside the agar. In swarm agar (0.5% and above), cells move as a group on the surface. While swarm plates must be used as detailed here, swim plates have a longer shelf life, and may be used for several days. Our personal preference is to use in 1-2 days

3.1. Preparation of swarm agar

3.1.1. Add 5 g of Eiken agar (0.5% w/v) and 20 g of LB to a 2 L round bottom flask. Add 1 L of ddH_2O to the flask and evenly mix the suspension using a stir rod and magnetic stirring plate.

3.1.3. Autoclave for 20 min at 121 °C.

3.1.4. Allow to cool with gentle agitation to avoid any air bubbles using the rod/plate as above. When approximately 50 °C, add filter-sterilized glucose for a final concentration of 0.5 %.

3.1.5. Pour 25 mL into sterile Petri dishes (100 mm x 15 mm) and allow to set at room temperature for at least 14 h and no more than 20 h. Do not store for future use.

3.2. Inoculation and incubation of swarm plates

3.2.1. Inoculate 6 µL of a mid-exponential culture (prepared as in 1.2) by spotting on top of the agar.

3.2.2. Leave the lid off for 5-10 min and replace when the inoculum has dried into the agar surface.

3.2.3. Incubate at 30 °C for 8 h. Avoid the temptation to inspect swarm progress by removing the lid, as this will contribute to drying of the agar and impair swarming.

NOTE: Incubation time may vary depending on the strain phenotype. Some isolated mutations may hamper swarming ability and will require a reduced percentage of agar or a more prolonged period of incubation.

3.3. Preparation of border-crossing assay plates

NOTE: This assay utilizes a modified Petri dish, where a plastic divide (border) creates two chambers, rather than one (**Figure 2A**). Each chamber can be prepared independently of the other, offering differing conditions for swarming, prior to 'connecting' the two. Depending on experimental design, the first chamber (designated left) can be prepared with either swim agar (0.3% w/v) or swarm agar (0.5% w/v) from where the bacteria can migrate across the border into the right chamber containing swarm agar +/- any required supplement or challenge (e.g., antibiotics). Migration on either agar is typically measured/compared by recording the widest diameter of bacterial colonization (edge to edge) from the original point of inoculation.

3.3.1. Prepare swim agar as described in 1.1 if required.

3.3.2. Prepare swarm agar as described in 3.1.5.

3.3.3. Pour ~30 mL of swarm agar (with desired supplementation if required) into the right chamber of a dual-compartment Petri dish (100 mm x 15 mm), to the point where it is level with the plastic divider between chambers, but not overflowing into the left (**Figure 2B**).

3.3.4. After the agar has hardened, fill the left chamber with ~30 mL of swim or swarm agar, again to the point of contact with the plastic divide (**Figure 2C**). Before it sets, use a sterile pipette tip to gently drag the agar over the border to connect the two sides with a ~1 mm tall agar bridge that spans the entire length of the divide (**Figure 2D**).

3.3.5. Allow the plate to dry at room temperature (3.1).

NOTE. An alternative method of creating bridge is allow the left chamber agar to dry and then slowly pipette ~100 µL of molten swarm agar along the plastic divider to bridge the two chambers (3.4). Inoculate the plates on the left chamber as detailed above for swim (1.2.2) or swarm (3.2) agar, before incubating at 30 °C for 12-16 h, or until swarms have made sufficient progress over the right chamber to allow comparisons between strains of interest.

REPRESENTATIVE RESULTS:

The isolation of pseudo-revertants in an *E. coli* strain whose motility is impaired by high levels of the signaling molecule c-di-GMP, was detailed in recent work from our lab[34]. This strain (JP1442) harbored two mutations: Δ*yhjH* and Δ*ycgR*. YhjH is the most active phosphodiesterase that degrades c-di-GMP in *E. coli*. Absence of YhjH leads to elevated c-di-GMP levels and inhibition of

motility. YcgR is a c-di-GMP effector. In complex with c-di-GMP, YcgR binds to the flagellar rotor to first induce CCW motor rotation and subsequently decrease motor speed. Cell tethering and bead assays showed that motor behavior returned to normal in the double mutant, yet motility in soft agar did not [34]. So, we deployed step 1 of the protocol to isolate pseudo-revertant flares in the double mutant (**Figure 1C**). The majority of the mutations mapped by WGS (HiSeq 4000 platform, PE 2 x 150 setup[34]) to *rssB*, which codes for a response regulator/adaptor protein that normally directs ClpXP protease to target σ^S for degradation[34]. One of these revertants, which displayed motility close to wild-type (AW405, compare **Figure 1A,D**), was used to generate representative results for step 2 and 3 of the protocol section, using as controls both its double mutant parent (**Figure 1B**) and isogenic wild-type strain (**Figure 1A**).

For step 2 of the protocol section, video captures were analyzed to calculate rotations per minute (each 360° complete rotation), and CW_{Bias} (the fraction of time motors rotate in a CW direction, or tumble bias). The Δ*yhjH* showed fewer rotations per minute and a lower CW bias compared to the wild-type, as expected (**Figure 3**). Both the Δ*yhjH* Δ*ycgR* double mutant and its suppressor showed motor behavior similar to wild-type, observations supported by a previous analysis using the higher-resolution 'bead' assay detailed in the introduction above in previous work[34].

For step 3 of the protocol section, the border-crossing assay (**Figure 2**) was used to compare the abilities of the wild-type and the suppressor isolate, first to swarm, and to then to move across the border and swarm on agar supplemented with kanamycin. Results show that both strains reached the border at a similar time (data not shown) indicating similar rates of swarming from an identical inoculation point. However, cross-over of the swarm to the right (antibiotic) chamber was marginally, but consistently greater for the wild-type than the suppressor at 20 µg/mL kanamycin (**Figure 4**). The difference between the two strains was more pronounced at 40 µg/mL kanamycin. Together, these data suggest that the mutations in *rssB* that restored motility on soft-agar plates (**Figure 1D**), negatively impact the antibiotic resistance of the suppressor strain during swarming (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Soft-agar motility assays and emergence of suppressor flares. The plates contain LB solidified with 0.3 % w/v agar. *E. coli* strains were inoculated in the center of each plate and incubated at 30°C for 8 h, except for C, which was incubated for 16 h. (**A**) Wild-type *E. coli* (AW405). (**B**) Motility-deficient variant Δ*yhjH* Δ*ycgR* (JP1442). (**C**) As in B, except longer incubation times. Arrows point to faster moving 'flares' emerging at the peripheral ring of the expanding swim colony. (**D**) A suppressor isolated from a flare in C.

Figure 2: Schematic for setting up a Border-crossing plate assay. (**A**) Pour ~30 mL of swarm agar (with desired antibiotic) into the right chamber of a divided petri dish until level with the plastic divider and allow to set with lid closed. (**B**) Fill the left chamber with ~30 mL of swim or swarm agar to the point of contact with the top of the plastic divider. (**C**) Use a sterile pipette tip to gently drag the molten swarm agar over the border, thereby connecting the two sides with a ~1 mm tall agar bridge and allow to set with lid closed. (**D**) Allow the plate to dry further at room

temperature overnight before inoculating the left chamber with the desired strain, and incubating at 30 °C.

Figure 3: Motor properties of various strains as measured by the cell-tethering technique. Wild-type (AW405), Δ*yhjH* (VN133), Δ*ycgR* Δ*yhjH* (JP1442), and its suppressor (JP1836) were grown in LB at 30 °C to mid-exponential phase prior to tethering. (**A**) Rotations per minute (completed 360° turns), and (**B**) CW_{bias} (fraction of time motors rotate in a CW direction). Standard deviation of the mean (±). 20 tethered cells were observed for 60 sec in each strain.

Figure 4: Border-crossing assays. Mid-exponential phase cultures of wild-type *E. coli* (AW405) and the suppressor mutant (JP1836) were inoculated at the indicated position (*) in the left compartment of the divided plate containing swarm media, and incubated at 30 °C. They reached the border at comparable times. The plates were incubated for a further 6 h, during which the swarm crossed over to the right chamber, in which the media was supplemented with kanamycin (Kan; numbers indicate μg/mL). Plates are representative of three biological replicates each carried out in triplicate.

Supplementary Figure 1: Preparation of a chamber slide for cell tethering. (**A**) Lay down two pieces of double-sided tape before (**B**) using a razor blade to trim away the excess. (**C**) Peel away the top layer to expose the adhesive before (**D**) affixing a coverslip and gently pressing it into position (indicated by **[**), ensuring all air is pushed out of the interface between the coverslip and tape below. (**E**) Load sample (shown here with DNA loading dye [30% v/v glycerol, 0.25% w/v bromophenol blue, and 0.25% w/v xylene cyanol] added to aid visualization) into the top of the created channel (arrow) while (**F**) angling onto clean, tissue task wipe to help draw the solution through the chamber as the tissue absorbs the liquid (arrow) in the channel and draws it through.

Video 1. Rotation of tethered *E. coli* cells.

Video 2. An active *E. coli* swarm filmed under 60x magnification, demonstrating its characteristic swirling motion behind the edge of the moving front.

DISCUSSION:

The isolation and characterization of suppressor mutations have successfully contributed to identifying key components of the chemotaxis system[35-37], as well as the motor machinery itself[38-40]. While using Protocol A, it is important to include multiple independent replicates to ensure the isolation of a large spectrum of possible mutations that could compensate for the loss of motility. Increasing the number of bacteria by streaking the culture in a line rather than a spot, can improve the odds of generating suppressors[41]. Isolation of the same mutation (as determined by DNA sequencing) multiple times increases confidence in its authenticity. WGS will invariably reveal the presence of other mutations in the genome. It is therefore important to verify the results by transducing (where possible) the identified mutation back into the original motility-deficient background. The suppressor mutant approach is rooted in restoring the function through a secondary mutation, so a limitation of this method is that if a critical structural gene is deleted, i.e., one that underpins the entire pathway or structure, there may be no scope for

compensation. Despite being an old method, our recent work[34] demonstrates its continuing utility in elucidation of new pathways that contribute to bacterial motility.

For the quantification of motor output, the cell-tethering approach remains a universally accessible tool requiring only a microscope with a camera attachment. Cell tethering has already been used in a diverse number of bacterial species including *Salmonella*[42], *Pseudomonas*[43], *Streptococcus*[44], and *Rhodobacter*[33]. The success of the protocol is largely contingent on proper shearing and attachment of cells. Shearing too aggressively or omitting the pause between shears (2.1.5) tends to promote inconsistent or incomplete shearing of filaments, resulting in non-motile cells or cells tethered on a skewed axis. The enduring relevance of this protocol remains, despite the adoption of the higher-resolution bead assay by many research groups (including ours). The primary limitation of the bead assay comes from the need for the bead to adhere to the filament of the bacteria of interest. This technique has greatly benefitted from studies in *E. coli* that identified a 'sticky' flagellin allele, which facilitates adherence[45]. The sticky variant is also superior in the cell-tethering assay. Such a variant is not yet available for the majority of flagellated bacteria. The situation is complicated further with some organisms possessing multiple flagellin proteins[46], and in the case of *Vibrio* sp., also possessing a membranous sheath[47]. Cell-tethering can also be performed using a species-specific anti-flagellin antibody or an antibody to an engineered epitope tag.

While bacteria can swim immediately upon introduction into a liquid medium, this is not true of swarming, where cells must be first primed into a swarming state. Surface contact triggers a physiological change required for cells to initiate swarming[48-51], resulting in a lag phase and a buildup of high cell density. Physiological changes include remodeling of the chemotaxis system in *E. coli*[16], and adaptations such as cell elongation and/or hyperflagellation for other bacteria[13,14,21]. Given the physiological changes required to initiate swarming, we strike a cautionary note about studies that have attempted to mimic select facets of swarming - such as high density or increased cell length – simply by concentrating planktonic cell cultures to increase density, and/or inducing cell elongation by use of antibiotics that inhibit cell division[52,53]. If using cell elongation as a marker, we caution also that compared to planktonic cells, there is only a marginal increase in the average length of swarmer cells in *Salmonella* or *E. coli*[54]. Swarm assays are harder to establish than swim assays. Variables include the commercial source of the agar used to solidify the media (the special Eiken agar is essential for swarming in *E. coli*, whereas the more standard Difco agar supports swarming for all other bacteria), use of rich versus minimal media (*E. coli* and *Salmonella* require glucose supplementation), and most critically ambient humidity[2,13,14,21]. Of these, maintaining optimal humidity can be the most frustrating. [An excellent, methodical, optimization for swarming in an organism of choice (*Pseudomonas aeruginosa*) has been demonstrated by Morales-Soto and co-workers[55].] Swarm media must be sufficiently moist to promote swarming but not so moist as to allow passive spreading/sliding, which can be readily mistaken for active swarming[56]. It is therefore critical that swarms be checked under a microscope to confirm the distinct patterns of movement associated with this collective motility (**Video 2**). Temperature is also an important consideration for optimizing the swarming assay. Higher temperatures, for example 37 °C, will dry out the plates sooner than at 30 °C. Using an incubator with humidity control (~70-80%) can help mitigate these issues,

including seasonal changes that could affect internal building temperatures and humidity. Once successfully established, protocol C provides a powerful way to investigate one of the most interesting aspects of swarming bacteria, elevated resistance to antibiotics[17]. All protocols described here can be applied to new organisms to identify pathways that specify and control flagella mediated motility.

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DISCLOSURES:

The authors have nothing to disclose.

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583

Figure 1

Click here to access/download;Figure;Figure 1.tif

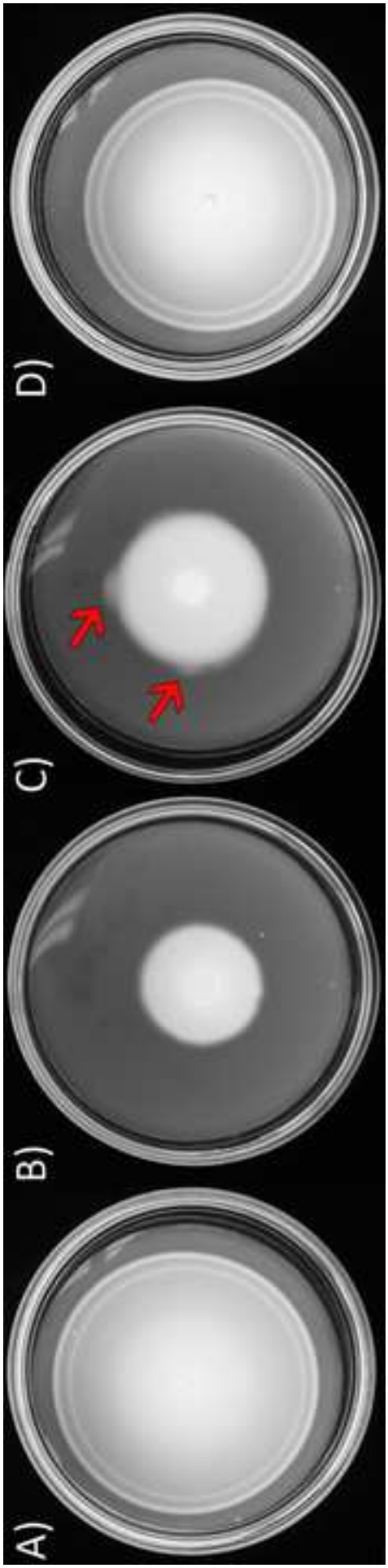

Figure 2

Click here to access/download;Figure;Figure 2.tif

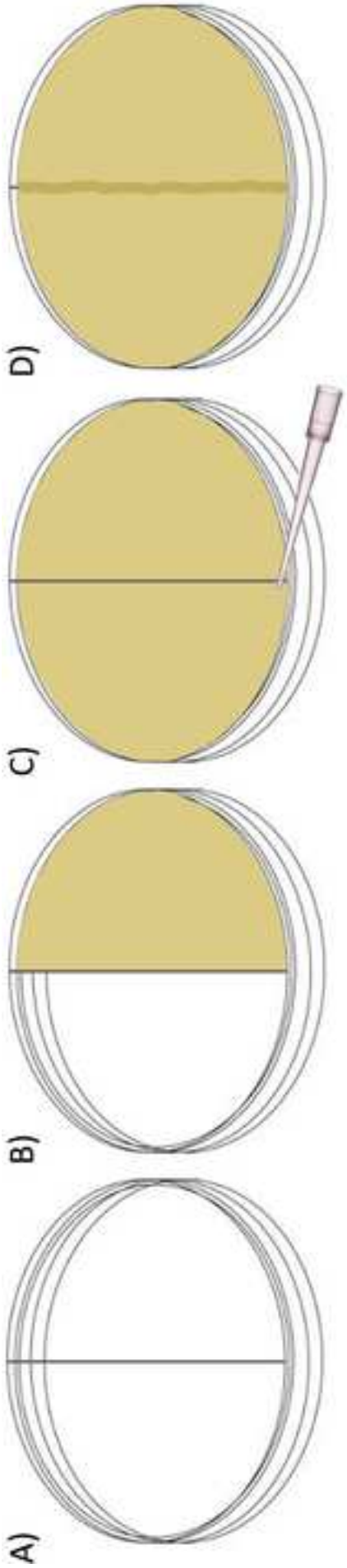

Figure 3

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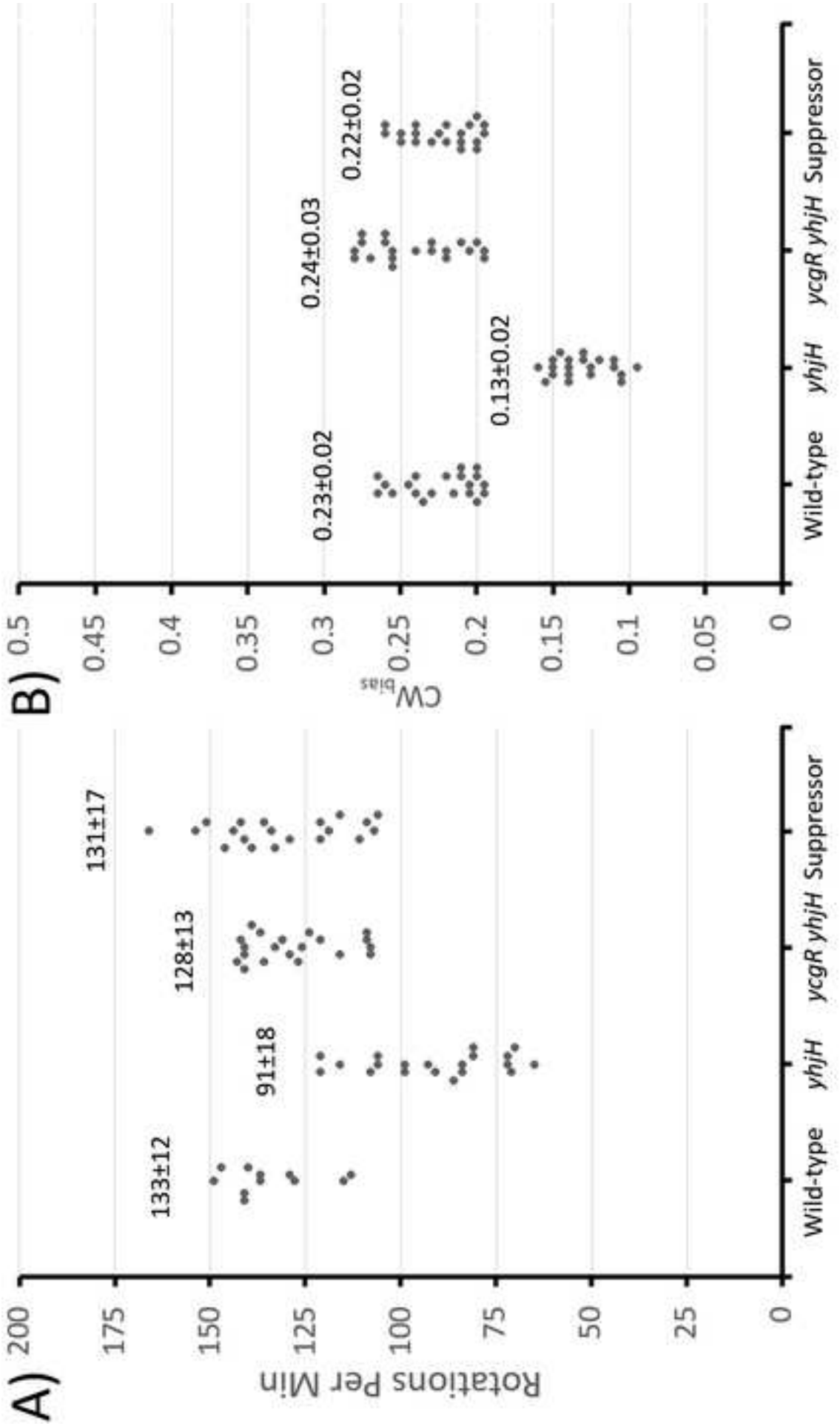
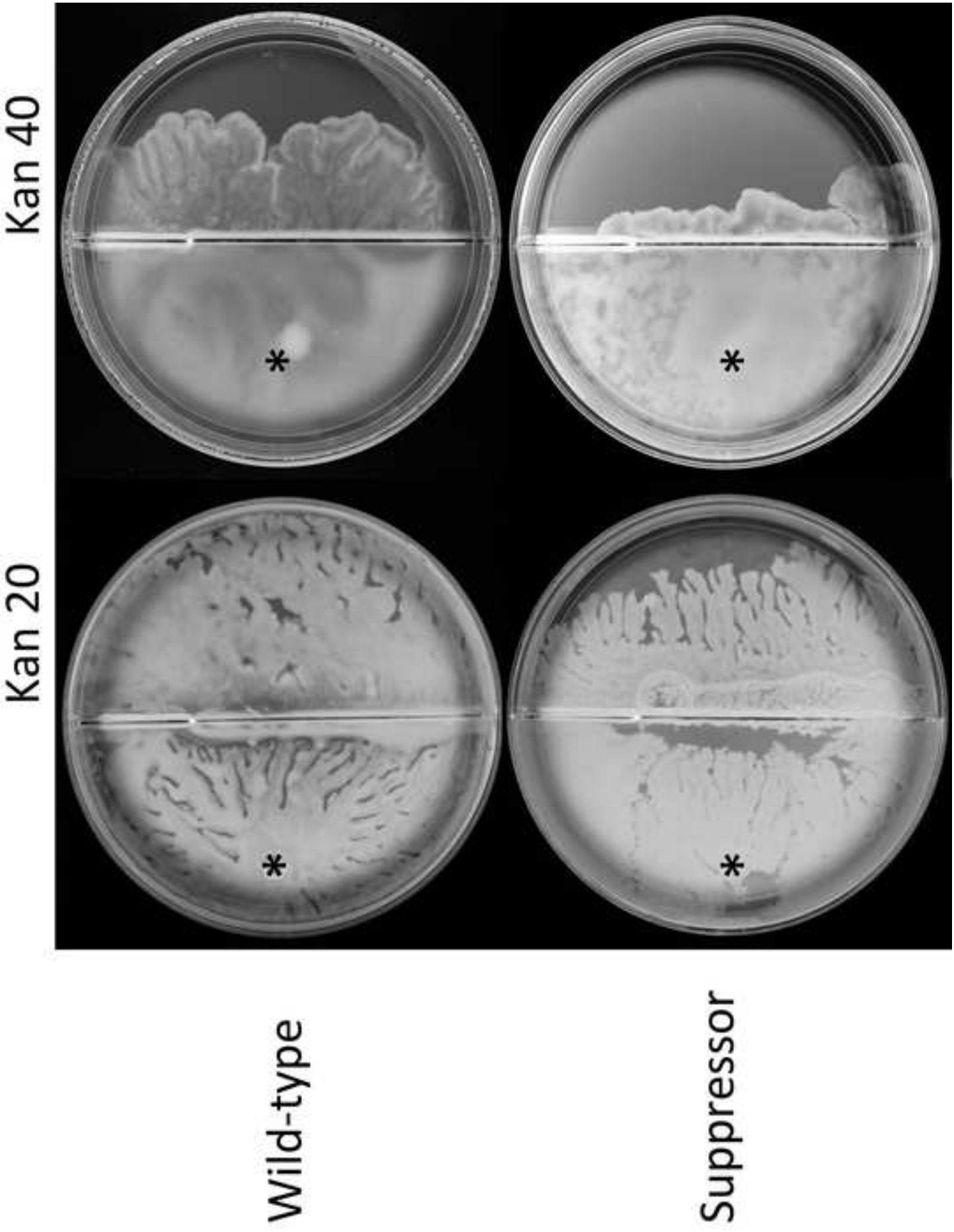


Figure 4

Click here to access/download;Figure;Figure 4.tif



Video 1

Click here to access/download

Video or Animated Figure

Video 1.m4v

Video 2

Click here to access/download

Video or Animated Figure

Video 2.m4v

Name	Company
Reagents	
Bacto Dehydrated Agar	Fisher Scientific
EDTA Disodium Salt, Dihydrate	Fisher Scientific
Eiken agar	Eiken Chemical Co. Japan
Glucose D (+)	Fisher Scientific
LB (Lennox) Broth	Fisher Scientific
Poly-L-lysine Solution (0.1%)	Sigma-Aldrich
Potassium chloride (KCl)	Fisher Scientific
Potassium Phosphate monobasic (KH_2PO_4)	Fisher Scientific
Potassium Phosphate dibasic (K_2HPO_4)	Fisher Scientific
Sodium chloride (NaCl)	Fisher Scientific
Materials and Equipment	
CellSense microscope imaging software (V. 1.6)	Olympus
Electron Microscopy Sciences Scotch 666 Doube Sided Tape	Fisher
Frosted microscope slides 3x1x1mm	Fisher
Olympus BX53 microscope	Olympus
Petri dishes (100 mm diameter)	Fisher Scientific
Polyethylene Nebulizer Capillary Tubing (0.58mm x 99mm 3.0m)	Perkin Elmer
Round Petri Dish with 2 Compartments	VWR
Safety Hypodermic Needles (23G)	Fisher Scientific
Sterile Syringe - 1 mL	Fisher scientific
Task/Tissue wipes	Fisher scientific
VWR micro cover-glass 18x18mm	VWR
XM10 camera	Olympus

Catalog Number	Comments
DF0140-15-4	
02-002-786	
E-MJ00	Essential for *E. coli* swarming
410955000	
BP1427-500	
P8920	
18-605-496	
BP362-500	
BP363-500	
S271-500	
	Or equivalent software for microscope used
50-285-28	
12-550-343	
BX53	Any upright or inverted phase microscope can be used
FB0875712	For soft-agar assays
9908265	
89200-944	For border-crossing assays
14-826A	
14-955-450	
06-666	Or equivalent single use tissue wipes
48366205	
XM10	Or equivalent microscope camera

We appreciate all the comments from the referees. Their feedback has helped refine and improve our manuscript. We hope you find the revisions, detailed point by point below, satisfactory.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Done

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Done

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Kim-Wipe, Olympus BX53 microscope, XM10 camera, CellSens software, VLC Player, etc

Done. Please note, due to the sensitivity of the swarm assay, it is essential that Eiken agar is used *for E. coli*, so it must be cited within the manuscript.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Done

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Done.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

No step contains more than 3 action sentences.

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Done. Note materials from Protocol C shifted into Discussion.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

More detail added, specifically to address the concerns of the referees below

9. Please ensure you answer the “how” question, i.e., how is the step performed?

See above

10. Lines 96-102: Please move the sentences where it is directly relevant with the steps.

Done.

11. 2.1: which strain is used in your case?

Detail clarified in representative results, line 255

12. 3.3: Please include how this is done? Please include all the button clicks in the software.

Text added to A-3.3, as per advice from correspondence with Vineeta Bajaj.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

All figures and data are new, we just use a strain characterized previously to build the representative results that use the series of protocols presented. So no copyright permission is required.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We do this, and the referees below confirm so.

16. Please do not abbreviate the journal titles in the references section.

We are using the JOVE template for Endnote so formatting should be correct. Correspondence with Vineeta Bajaj advised us to leave the formatting as is.

17. Please sort the Materials Table in alphabetical order.

Done

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

In the present manuscript, Partridge and Harshey describe three classical techniques to study the motility behavior of bacteria with a focus on Escherichia coli.

The presented techniques are well described and I only have few minor comments:

Part A. Isolation of suppressor mutants

1) In addition to the described unbiased suppressor mutant selection, a targeted mutagenesis strategy (e.g. PCR mutagenesis) can be used.

Added, see line 73

2) Inoculating a culture in a 'spot' for selecting of suppressor mutants works, but limits the number of

bacteria at the inoculation point. Another approach is to 'streak' the culture in a line, compare e.g. Figure 1 of Barker et al. PLoS Genet 6, e1001143 (2010).

Added. See line 329.

3) Purified suppressor mutants might be re-transduced in a clean, motility-deficient background selecting for Mot+ in order to separate potential unrelated mutations from the wanted suppressor mutation and facilitate interpretation of the whole genome sequencing results.

Added, line 333

4) Whole genome sequencing might not be necessary in all cases, e.g. if targeted mutagenesis has been performed.

Added “as required” to line 1415

Part B. Quantifying Flagella Motor Behavior via Cell Tethering

1) Why mix imperial with metric units?

We state the speed as g (RCF) as rotor type/size can give different results if following RPM. Citing ‘g’ offers the better chance for readers to accurately reproduce conditions.

2) Different approaches to tether cells to a surface, e.g. using anti-flagellin antibodies should be discussed.

Added, line 353

3) In support of Video 1, some additional and more detailed description of the rotation analysis of tethered cells might be useful. What does 'rotating on a single axis' mean?

Clarified, line 178

4) Automated software for rotation analyses are also available and should be discussed (e.g. PMID 21515726)

Note and reference added, line 187

5) Details concerning the image capture settings would be appropriate (e.g. how many frames per second are needed? Which magnification is appropriate?).

Some details already present in line 177. Added a note on fps, line 182.

Part C. Preparation of swarms in a border-crossing assay

1) Ordering details for the dual-compartment petri dishes should be provided

Listed in table of materials

2) It would be useful to describe possible approaches to quantify swimming or swarming motility
Added, line 224

Reviewer #2:

Manuscript Summary:

Partridge and Harshey have a very well written, referenced, and detailed account of a common technique in the field. The introduction is particularly concise and summarises while this technique may be old, it is still used widely.

I strongly recommend this for acceptance in JOVE with no major concerns. Minor concerns and reference suggestions below.

Minor Concerns:

L25: 'Everything old is new again'. Maybe this is a quote from somewhere, eg Peter Allen song, but it is a little out of place here could probably just omit it (sorry to be a killjoy but on my first read was just wondering what was going on).

Deleted

L32. Perhaps the definition here of gain-of-function supressor could be expanded or said more simply here where it first appears. I understand what is implied but gain-of-function suppressor seems contradictory, perhaps could just have an additional clause, ie 'where motility is restored through some mutation', or some such.

Added, line 32

L47: An extra suggested ref for virulence:
https://onlinelibrary.wiley.com/doi/full/10.1002/jobm.201100335
Duan et al. Journal of Basic Microbiology 2012.

Added

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3165335/
Rasmussen et al. Antimicrob Agents Chemother. 2011.

Not relevant

L50: An extra suggested ref for ion gradients and torque generation:
https://jb.asm.org/content/202/3/e00557-19/article-info
Lai et al. J. Bact 2020
Not relevant

L50: An extra suggested ref for torque speed relationship of BFM, more contemporary:
https://www.pnas.org/content/103/5/1260 (2006)
Xing et al. PNAS 2006

Added

https://www.pnas.org/content/114/44/11603 (2017)
Nord et al PNAS 2017

Not relevant/necessary

L63: swarm only _in_ media solidified with...

Fixed

L64 lose 'even', ie, can navigate across higher agar concentrations.

Fixed

L76: Add PNAS 2017 ref from above in reference to advanced bead/gold assays:
https://www.pnas.org/content/114/44/11603 (2017)

Ref 21 (Ryu et al) removed, suggested ref added where requested

L76. I am not sure whether this should be 'flagellar filaments', or just flagella. I prefer here 'flagella', as that is what we are shearing. It depends what you want to say.

Fixed, line 81

L109: Can you comment on whether swim plates can be used after more than 16 hrs?

Added details, line 197.

L129: Can you be more specific on how you determine the edge of the swim circle in order to make a measurement? Are their contrast settings or such in the image, or some method to determine where edge of swim circle is?
Added, line 98

L133: What type of sequencing do you do here? What coverage? Why WGS? Can you include some methods line and reference for the WGS that you suggest to do here.

The approaches and platforms available are varied, and as such we prefer not to specify in the protocol. We have added advice (lines 141 and 247) and a reference in the representative results to our WGS approach.

L146: Interesting, I did not know this about pauses - a useful contribution to the literature.

Thank you!

L299: Suggest adding reference for some other recent suppressor mutation work:
https://onlinelibrary.wiley.com/doi/full/10.1111/mmi.14246
Ishida et al. Mol. Micro 2019
Added

Reviewer #3:

Manuscript Summary:
This is a nice useful outline of protocols for assaying flagellar function, taking advantage of the selective benefit of motility that will be invaluable to newcomers to the field. The writing is overall clear (although see below) and figures illustrate the paper well. The videos nicely illustrate key results.

Major Concerns:
I have no major points to make about this work.

Minor Concerns:

* "Everything old is new again" sounds catchy, but it's not clear what it's referring to.

Deleted

* Writing is unneccesarily wordy and would benefit from a thorough edit (for example, the abstract

includes superfluous words or phrases, such as "as a means", "in a series", "strong", "readily"; "signalling effectors" is redundant, etc. But this continues throughout the manuscript).

Manuscript edited for brevity

* Readers may benefit from a brief description as to the typical circumstances in which they would choose each assay.

Text added at the beginning of protocols A, B, and C.

* Because this is likely to be an important guide for newcomers to the field, I'd recommend more fully explaning a few aspects of the systems described — I can imagine a new PhD student finding various aspects confusing without further explanation. Line 145, 176: these are really nice examples of explanations to your readers, more of this would be great. Spell it out for those poor new graduate students!

More explanations have been seeded throughout the text, primarily due to addressing other referee comments, notably #1.

* Readers unfamiliar with the system would benefit from a more explicit explanation of how CW/CCW rotation influence runs and tumbles.

Expanded, lines 51-58.

* Line 93: If the readers are advised to use a bucket of light, I'd recommend including a paragraph describing it instead of making readers refer to the reference.

Description/reasoning added, line 99

* Line 94: Readers may appreciate clarification that chemotactic-deficient strains and motility-deficient strains behave similarly in this assay.

Added, line 107

* Line 94: Highlight that can be used for more than just suppressor identification (e.g, transposon screens, etc).

Added, see line 73

* Line 112: Readers may appreciate an explanation of why 30 °C and not 37 °C.

Added, line 375

* Line 134: Sequencing alone is insufficient. Mutants should also be confirmed by reconstitution in the parental strain!

Added a line on verification in a ‘clean’ background, line 333

* Line 138: Explain to readers why MB not LB.

Added, line 152

* Line 153: Highlight that polylysine is just one way of tethering a cell.

Added, line 352

* Line 169: Highlight that increasing the viscosity of the medium may help resolve rotation direction, especially with a cheap low framerate camera. With species other than E. coli this may be particularly important.

Added, line 188.

* Line 175: Would benefit from an overview of the assay before launching into it.

Added, line 207

* Line 189: I imagine this depends upon ambient humidity — 14-20h may be good in Texas, how about in California, Scandinavia, Australia?

This is discussed in line 356

* Line 201: Without having introduced the assay, it isn't clear what the "first compartment" is. Needs introduction earlier.

Added, line 218

* Line 210: make explicit how side the bridge is: also ~1mm, or along the entire length of the divide?

Added, line 235

* Paragraph line 221: Would benefit from a little more unpacking and explanation.

Added notes to better connect setup to the schematic figure. Further clarification will come from the video recording.

* Paragraph Line 234: Potential users would benefit from knowing the camera type/framerate

Added to table of materials and line 182

* Line 320: emphasis that the reader will need to develop novel approaches to tethering, and highlight plausible and less plausible possibilities (e.g., affinity tags, lectins, antibodies, glow discharge, etc).

Added, line 353

* Figure 1: Add arrowheads to call out flares?

Added

* Figure 4: belongs before Figure 3?

Switched!

* Figure 4: images don't correspond to labels? Can you add a 'sterile pipette tip' to the schematic?

Labels removed as per reviewer 4, sterile tip added

Reviewer #4:

This manuscript provides a whole workflow of three standard bacterial motility assays starting with the isolation of a suppressor mutant in motility-deficient strains and going on with possible characterization techniques to investigate its impact on motility. A) The capability to spread in soft-agar is used as an easy way to select for suppressor mutants and to compare swimming motility of the wild type, a motility-impaired mutant and its suppressor mutant. B) By cell tethering, the motility is characterized at the motor level. The motor speed and rate of switching rotational direction is measured. C) With the border crossing assay swarming efficiency under different conditions can be compared. This paper comprises detailed protocols for all three methods and proves its application by representative results of their own work.

In the protocol A (isolation of mutants) it becomes not completely clear from where exactly the suppressor mutants are isolated. The motility rings spreading form the inoculation point but also the ones outgrowing from the periphery both termed ´flare´ region. Under 2.3. the instruction is given to isolate colonies from the ´flare´ region. Here, only the second type of ´flare´ regions, where additional motility circles arises from the periphery, are meant. This should be pointed out more, e. g. by an arrow in Figure 1C or by further explanation in the text.
Arrows added to figure

Furthermore, in line 131 authors refer to a figure 1E which does not exist.

Deleted

In the protocols each step is explained in detail, so that it can be reproduced easily. For the sake of completeness, it should also be mentioned that the glucose used for swarm agar has to be sterile-filtered (line 188).

Added

The representative results originate from recently published data by the authors. The citation is enough to justify the statements. Further connections to the paper, as the link to specific figures, are not necessary for understanding the presented protocol. Moreover, the paper is not accessible to everyone.

Reference to specific figures deleted.

In the abstract and introduction, the border-crossing assay is introduced as a method to improve the swarming motility by inoculating the bacteria first in swimming agar and priming transition into swarming at the border of the two compartments. When reading the protocol for preparation of border-crossing assay plates, it is not clear why swarming agar could also be used for the first compartment and what is meant by ´required supplement or challenge´. Only later the representative results and the discussion reveal a great application to validate the impact of e.g. antibiotics on swarming. This should be mentioned earlier, either in the introduction or by adding an example in brackets behind ´required supplement or challenge´. Do also other supplements or challenges were tested by this setup? Maybe some more examples can be named.

A 'lead in' to the protocol has now been added (line 218) as well as the suggestion to add an example.

In Figure 3 the inoculation position is marked for the wildtype. Same should be done for the mutant, so that the reader can directly see that it needs to be at the same position with the same distance to the border.

Added to all panels, figure is now Fig. 4

Figure 4 illustrates how to prepare the border crossing assay. The explanation for this is given in the protocol, in the figure itself and in the caption of the figure. It should be left out either in the figure or in its caption.

Labels removed from figure (now fig 3).

Some minor errors/ misspelling
* check headings (uniform capitalization)

Attended to

* line 102, 237: remove period

Added

* line 103: 3_g, add space

Added

* line 172: it must be 3.4. instead of 3.3.

Fixed

* from line 200: wrong numeration, instructions should get numbers starting with 3.1 not 1.1

Fixed

* line 202: close bracket after ´0.5% w/v ´

Added

* line 214: cross reference C.1.5 would be more precise

Added

* line 222, 225: c-di-GMP, the ´i´ is missing

Added

* line 329: delete ´) ´

Deleted

All in all I think this manuscript is nicely written. It consists of three methods which can be easily established in every lab without the need of expensive equipment. Furthermore, the manuscript gives multiple approaches to troubleshoot problems in the presented techniques. Although each method itself is well known, the combination of these techniques provides a fast and easy access to study components of the motility pathway in different bacteria.

Supplementary figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1.tif