**Motility assays: twitching motility**

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## i. Summary

Twitching motility is a mode of solid surface translocation that occurs under humid conditions on semi-solid or solid surfaces, is dependent on the presence of retractile type IV pili and is independent of the presence of a flagellum. Surface translocation via twitching motility is powered by the extension and retraction of type IV pili and can manifest as a complex multicellular collective behavior that mediates the active expansion of colonies cultured on the surface of solidified nutrient media, and of interstitial colonies that are cultured at the interface between solidified nutrient media and an abiotic material such as the base of a petri dish or a glass coverslip. Here we describe two methods for assaying twitching motility mediated interstitial colony expansion in *P. aeruginosa*. The first method, the "Macroscopic Twitching Assay", can be used to determine if a strain is capable of twitching motility mediated interstitial colony expansion and can also be used to quantitatively assess the influence of mutation or environmental signals on this process. The second method, the "Microscopic Twitching Assay", can be used for detailed interrogation of the

movements of individual cells or small groups of bacteria during twitching motility mediated colony expansion.

**ii. Key Words**

Twitching motility, type IV pili, surface motility, tfp, fimbriae, interstitial colony, interstitial biofilm

## 1. Introduction

*Pseudomonas aeruginosa* undergoes a form of surface translocation known as twitching motility. This motility is independent of the flagellum but is dependent upon the presence of retractile type IV pili (tfp), also called type IV fimbriae. The tfp are polar filamentous appendages of 5-6 nm in diameter and are of average length of 1-4 μm but can be up to 10 μm long. The tfp are composed of several thousand copies of a single subunit protein termed pilin or PilA, arranged in a helical symmetry of 4 subunits per turn. The production of tfp is dependent on the culture conditions, being produced during plate or stationary broth culture but not during vigorously shaken broth culture (for recent reviews see [1-3]).

When cultured in humid conditions on the surface of solidified nutrient media, colonies of *P. aeruginosa* that are capable of twitching motility grow as flat, rough, spreading colonies with a characteristic “ground glass” edge. Strains that lack functional tfp or are no longer capable of twitching motility produce smooth, domed colonies that do not actively expand across the surface via twitching

motility [4, 5]. Whilst the obvious distinction in these phenotypes has been utilised in genetic screens to identify genes involved in twitching motility [6], the observation of differences in colony morphology provides little insight into the process by which twitching motility mediates active colony expansion. More sensitive assays of twitching motility were developed when it was observed that *P. aeruginosa* exhibits extremely active twitching motility mediated colony expansion when cultured at the interstitial surface between agar and the base of a plastic petri plate [4, 7]. When a small inoculum of *P. aeruginosa* strains that are capable of twitching motility are stabbed through a layer of agar to the underlying plastic*,* a large halo of interstitial colony expansion is obtained after overnight incubation, whereas non-twitching strains produce no such zone of colony expansion ([4]; see **Fig 1**). This “Macroscopic Twitching Assay” is now used as a *de facto* measurement of twitching motility activity and can be modified easily to observe the effect of different environmental stimuli by simply adding compounds to the solidified nutrient media.

However, the “Macroscopic Twitching Assay” does not allow detailed analyses of individual cell movements during interstitial colony expansion. High resolution microscopy of twitching motility mediated colony expansion is possible using a thin layer of solidified nutrient media and a glass coverslip to create the interstitial interface at which active colony expansion occurs [4]. Microscopic observation of the interstitial colony created at the glass/media interface reveals that under these conditions, twitching motility manifests as a highly organised

multicellular collective behavior that enables rapid colonisation of large areas and involves the formation of an intricate lattice network of cells behind outgoing leading edge rafts of cells ([4]; see **Fig 2**).

In this chapter we describe these two methods for assaying twitching motility in *P. aeruginosa*. The “Macroscopic Twitching Assay” is suitable for quantitative medium throughput screening of twitching motility ability on a macroscopic level. This method may be used for simple determination of the ability of a strain to demonstrate twitching motility mediated interstitial colony expansion or may be performed with replicates to allow quantitative determination of the effect of mutation or environmental changes. Suitable positive control strains for this assay include PA01, PA103, PA14 or PAK. A suitable negative control strain would be an isogenic mutant that is unable to assemble functional tfp such as a mutant of the major pilin subunit gene *pilA*.

The second method, the “Microscopic Twitching Assay”, allows microscopic examination of collective and individual behaviors of bacteria undergoing twitching motility mediated interstitial colony expansion. The “Microscopic Twitching Assay” described below is optimised for obtaining a layer of single cells at the leading edge of the colony to allow high resolution microscopy and tracking of individual cell movements when time-series are captured. In this

assay the nutrient media is solidified with a gellan gum, which provides greater optical clarity than agar.

## 2. Materials.

Prepare all solutions using ultrapure water (prepared by purifying deionised water to attain a sensitivity of 18 MÙ cm at 25°C) and analytical grade reagents. Chemicals specific to these protocols are listed below. Prepare and store all reagents at room temperature (unless indicated otherwise).

### 2.1 Macroscopic Twitching Assay

1. 1% LB-Lennox agar (1%LBA): 4 g Tryptone, 2 g yeast extract, 2 g NaCl, 4 g agar (see **Note 1**), 400 mL water.
2. Petri plates: sterile, 90 mm x 10 mm (see **Note 2**)
3. Autoclavable bottle, minimum volume 500 mL (see **Note 3**)
4. Waterbath set to 55°C
5. Disposable 25 mL sterile serological pipettes
6. Automatic pipettor
7. Wooden toothpicks or yellow plastic disposable pipette tips (see **Note 4**)
8. Plastic disposable sterile inoculation loop, 10 μL
9. Humidified chamber: Plastic or glass lunchbox/container with lid containing paper towel in base dampened with sterile water.

10. TM developer solution: 400 mL water, 100 mL glacial acetic acid, 500 mL methanol, store at 4°C

## 2.2 Microscopic Twitching assay

1. Twitching motility Gellan Gum media (TMGG): 0.8 g gellan gum (see **Note 5**), 0.4 g tryptone, 0.2 g yeast extract, 0.2 g NaCl, 0.1 g $MgSO_4.7H_2O$, 100 mL water
2. Dry heating block set to 65°C with holder for 50 mL tubes or a dry heating bath containing metal beads set to 65°C (see **Note 6**).
3. Heated slide preparation stage set to 65°C (see **Note 7**).
4. Large petri plates (145 mm diameter), sterile
5. 50 mL disposable plastic tubes, sterile
6. 5 mL disposable plastic tubes, sterile
7. Forceps, metal
8. Microscope slides (75 x 25 mm)
9. Coverslips (22 x 40 mm) (see **Note 8**)
10. Spatula, fine
11. Disposable plastic inoculation loops, 10 μL
12. Tissues (see **Note 9**)
13. Lint free cleaning tissues (Kimwipes)
14. Paraffin film (Parafilm)

15. Humidified chamber: Plastic or glass lunchbox/container with lid containing paper towel in base dampened with sterile water sized to hold large petri dish.

## 3. Methods

### 3.1 Macroscopic Twitching Assay

All procedures are performed at room temperature unless otherwise specified. The following protocol will make 40 assay plates. Scale the volume and components accordingly to make the number of plates that you require.

1. Streak each strain to be assayed onto a 1.5%LBA media plate and incubate overnight at 37°C.
2. Add tryptone, yeast extract and NaCl to 400 mL water and mix until fully dissolved.
3. Add agar to autoclavable bottle.
4. Add dissolved medium to agar bottle and gently mix to avoid agar clumping. Agar will not dissolve.
5. Autoclave to yield a sterile homogenous suspension (20 min at 121°C, 15 psi should be sufficient).
6. Place autoclaved 1%LBA in heated waterbath and allow to cool until medium is at 55°C.
7. Place 40 petri plates on bench in a single layer.

8. Using a 25 mL disposable serological pipette attached to an automatic pipettor, dispense 10 mL of molten 1%LBA into each petri plate. If volume does not cover bottom of petri plate, swirl gently to spread evenly.
9. Leave agar to set for 1 h.
10. Invert petri plates on the bench and leave overnight to dry in a single layer (see **Note 10**).
11. The following day, label the base of each plate appropriately.
12. Remove inoculation plates from 37°C incubation. With a plastic inoculation loop take a small portion of the outer edge of the bacterial streak and gently mix in a sterile area of agar until the bacterial culture is smooth (see **Note 11**).
13. Use a sterile toothpick or sterile yellow pipette tip to scoop a match-head sized inoculum from the mixed culture. Try to keep the inoculum size similar for each plate.
14. In the center of each 1%LBA plate, stab the inoculum perpendicular to the agar down to the agar-plastic interface at the bottom of the plate. Jiggle the toothpick/tip up and down a few times to ensure the inoculum has reached the bottom of plate. Do not move toothpick from side-to-side (see **Note 12**).
15. Invert plates into a humidified chamber in stacks of no more than 3 plates high.

16. Place in standard 37°C incubator with lid slightly ajar to allow air to enter the humidified chamber. Incubate in humidified environment at 37°C for 24 h.
17. Remove petri plates from incubator (see **Note 13**).
18. There are two types of colony growth on the petri plate. There will be a colony on the surface of the agar around the inoculation point (top colony) and a visible halo of bacteria that have twitched across the plate between the bottom of the agar and the plastic petri plate (interstitial colony). The interstitial colony can be seen by holding the plate up to a light source (see **Note 14**). Mark each edge of interstitial colony with a permanent marker ("Sharpie") (see **Fig. 1A)**.
19. The surface area of the interstitial colony can be calculated as follows. If the interstitial colony is circular in shape then the surface area can be calculated using the formula $\pi r^2$ where r = ½ the diameter of the interstitial colony. If the interstitial colony is oval in shape, measure the longest and shortest diameters and calculate the surface area by using the formula π x a x b where a = ½ longest diameter and b = ½ shortest diameter.
20. To visualise the interstitial colonies for image capture or to aid visualisation for measurement, flood the plates with a small amount of cold TM developer solution. The solution makes the interstitial colony appear as an opaque white halo (see **Fig. 1B**).

21. Leave the TM developer solution on the petri plates until the interstitial colony is visible as a white halo or for up to 30 min (see **Note 15**).
22. Using a plastic disposable loop, gently scrape the top colony away from the agar, being careful not to scratch the surface of the agar.
23. Carefully decant the solution from the plates into a beaker. Make sure the outsides of the plates are dry and clean by wiping with a tissue if necessary.
24. Images of the interstitial colonies can be recorded by scanning the petri plates using a flatbed document scanner (**Fig. 1**). It is useful to draw a 1 cm line on the base of the petri plate prior to scanning for use as a scale bar.

### 3.2 Microscopic Twitching assay

1. Weigh out each component of TMGG except the gellan gum and add to water in a beaker containing a magnetic stirring bar.
2. Heat the medium with stirring on a magnetic stirring/heating block.
3. Gradually add the gellan gum in small amounts with stirring to prevent clumps forming. Heat the medium to boiling while stirring to dissolve.
4. Once the mixture turns clear, autoclave for 20 min at 121°C, 15 psi (see **Note 16**).
5. Remove sterile TMGG from autoclave and decant into 50 mL tubes that have been pre-heated to 65°C either in a dry block heater or in the metal beads of a 65°C dry bath. Allow to cool to 65°C. Gellan gum will set

below 60°C, so care must be taken to keep temperature of TMGG above 65°C. All procedures should be done as quickly as possible to avoid TMGG setting in tubes. Care should also be taken to minimise water loss by steam or condensation and by minimising the time that lids are removed from containers (see **Note 17**).

6. Pre-heat the slide preparation stage to 65°C, and ensure it is level (see **Note 18**).
7. Pre-heat 5 mL tubes to 65°C in the heating block or dry bath.
8. Pre-heat a large petri plate on the 65°C warm stage.
9. Flame sterilise 4 microscope slides by holding each slide with a pair of forceps, dipping the slide into a glass beaker containing 96% ethanol, touching the corner of the slide to the edge of beaker to drain off most of the ethanol and passing the slide through the flame of a Bunsen burner to burn the remaining ethanol. Align each slide side by side inside the petri plate using sterile forceps. Push the slides tightly together so that the long edge of one slide is touching the wall of the petri plate and allow to pre-heat to 65°C (see **Fig. 3A**).
10. Decant 5 mL of TMGG into the pre-warmed 5 mL tube. Return the 5 mL tube to heating block or dry bath to maintain temperature.
11. Remove lid of petri plate and rest on bench so that you have 2 free hands.
12. Remove 5 mL tube with TMGG from heating block and pour TMGG over all four slides. Gently tilt the petri dish from side to side so that the TMGG

covers all surfaces of the slides. Try to get an even coverage of TMGG on the 4 slides but not let the TMGG pour off the edges of the slides. Some TMGG may leak between the slides and settle between the slides and the bottom of the petri plate; this is acceptable.

13. Allow TMGG to settle, replace lid and move petri plate carefully from the warm stage to a cool, level area and allow TMGG to set for 15 min.
14. Fold a tissue in half and in half again until you have a small wad approximately 1 x 4 cm in size. You will need two per petri plate. Wet the tissues with sterile water until the tissues are full but not quite dripping and place at empty ends of the petri dish. Do not let free water run into the base of the petri plate. Be careful not to let the tissues touch the slides (see **Fig. 3B**).
15. Holding the petri plate level, seal the lid to the base using paraffin film and transfer to a 4°C refrigerator until required (see **Note 19**).
16. Streak the desired strain(s) to be assayed onto a 1.5%LBA plate and incubate overnight at 37°C.
17. The following morning, remove the pre-poured slides from 4°C.
18. Remove the two tissues from each end of the plate and keep aside aseptically.
19. Flame sterilise a fine spatula and use to gently score the TMGG between each slide and separate slides a little using the spatula.

20. Place open large petri plate (lid removed) in a Type 1 or Type 2 Biohazard Cabinet for approximately 20 min to dry slides (use the same cabinet each time for consistency, see **Note 20**).
21. Remove petri plate from cabinet and remove excess TMGG from under the slides. To do this, slowly lift out each slide using a spatula and wipe the back of each slide with a lint free cleaning tissue. Also wipe out the petri plate. Replace slides into the large petri plate.
22. Using the edge of a flame-sterilised fine spatula, gently score around the edge of each slide. Remove excess TMGG by lifting gently away with the flat edge of the spatula. Trim 1-2 mm from the long edges of the slide, 5-10 mm from the clear short edges to prevent contact of the TMGG with the microscope stage and all of the white section so that slide can be adequately labeled (see **Fig. 4A**).
23. With a plastic inoculation loop take a small portion of the outer edge of the bacterial streak and gently mix in a sterile area of agar until the bacterial culture is smooth (see **Note 11**). Collect a small amount of culture on the tip of the loop and lightly dab onto the center of the TMGG slide, creating a small flat inoculation point of 1-2 mm in diameter.
24. Using 96% ethanol, flame sterilise a 22 x 40 mm coverslip and allow to cool. Placing one short edge of coverslip onto the TMGG, slowly lower coverslip onto the TMGG at an angle using either sterilised metal forceps or spatula, trying to minimise bubble formation between the coverslip and

the TMGG (see **Fig. 4B**). Repeat Steps 23 and 24 for all slides (see **Note 21**).

25. Place slides back into petri dish along with damp tissues, being careful not to let the tissues touch the slides (to prevent excess moisture being introduced under the coverslip).
26. Incubate at 37°C for at least 4 hours, until required for microscopic analysis. The resulting interstitial colony can be visualised by phase contrast microscopy at high or low magnification (see **Fig. 2**; **Note 22**). Series of time-lapse images can also be captured to create a movie of twitching motility.

## 4. Notes

**1.** Agar suppliers: The brand of agar used can significantly affect the results of this assay. It is recommended that you acquire some samples of agar from different suppliers and test in this assay. Some brands of agar may even inhibit twitching motility. For testing with 1%LBA, you should be able to see a 3-5 cm diameter interstitial colony at 24 h with a wildtype strain such as PAO1, PAK or PA103. We use agar No. 1 obtained from Oxoid in our laboratory.

**2.** Petri plates: We use standard sterile petri plates available from most bulk suppliers but again some are better than others for enhancing twitching motility. We note that tissue-culture treated petri plates will dramatically enhance twitching motility mediated interstitial colony expansion. If we have an

ambiguous result with a strain, we will use a tissue-culture treated petri plate as a follow up to increase the sensitivity of the assay.

**3.** It is advisable to set aside a stock of bottles that are only used for making TM assay media. Results will be more reproducible if you minimise the possibility of chemical contamination into the bottles.

**4.** We usually choose the cheapest toothpicks at a supermarket. These tend to be the best for this assay. Some brands are impregnated with antimicrobial compounds and should be avoided.

**5.** Gellan gum is available under the trade names Gel-Gro, Gelzan and Gelrite. Gellan gum is optically clearer than agar as a setting agent and does not auto-fluoresce. This reduces background for both phase-contrast and fluorescence microscopy and enables the addition of fluorescent dyes to the TMGG or to use bacteria expressing fluorescent proteins for the assay.

**6.** We do not recommend the use of water baths for this method. We have found that the need for rapid movements during this method can lead to contamination. Dry heating is best.

**7.** We use a heated slide preparation stage for this step. It is the best option if you have access to one. However, you can also use a dry block heater with the blocks turned upside down to maximise the heating surface. It is very important that you can control the temperature so we do not recommend a normal heating plate unless it has a temperature control feature.

**8.** Coverslip and slides: We have found that some batches of slides and coverslips can be covered in debris. If this happens, we recommend cleaning

the slides with a toothbrush and powder cleaner such as Ajax and rinsing well with deionised water. Coverslip can be cleaned also with a powder cleaner by rubbing gently between thumb and forefinger, taking great care not the crack the coverslip or cut the operator. If you are observing the twitching motility with phase contrast microscopy, you may use #1 coverslips (0.13-0.16mm thick). However, if you are going to perform high resolution fluorescence microscopy, we recommend the use of #1.5 coverslips (0.17 mm thick) for optimal imaging.

**9.** Tissues**:** We have found that occasionally tissues can be a source of contamination. If you are experiencing contamination in your assay, make a batch of wet tissues, autoclave and keep aseptically until needed.

**10.** It is important to dry plates so that no water is visible on the surface but not to over-dry them. If the conditions are too moist, it is possible that the bacteria will swarm or swim instead of twitch and if the plates are too dry, twitching will be inhibited.

**11.** Homogeneous inoculation culture: as the area of most active twitching motility is located at the outermost edge of the colony, we have found that in order to make the assay as consistent as possible (especially if doing large numbers of assays from the one streak plate), it is best to mix by swirling with a plastic inoculation loop, a small portion of the outer active edge of the plate culture until smooth and homogeneous and use this for all replicate inoculations. We do not recommend using liquid overnight cultures for inoculation as flagellated strains will swim or swarm if too much liquid is deposited in the stab site. Although the liquid will eventually dry out at the

inoculation site and the strains will then twitch, the rapid swimming or swarming may give a false positive or a falsely enlarged interstitial twitching colony.

**12.** It is important to keep the toothpick or pipette tip as upright as possible during the inoculation. Make sure that you have been able to push some bacterial culture to the bottom of the plate by turning the petri plate upside down and visually inspecting. It is also important during this step to not leave a large mound of bacterial inoculation culture on the top of the agar to form a large top colony. If the top colony grows too large, it may inhibit interstitial colony expansion and you will get skewed results. For all initial tests of new strains or environmental stimuli, we recommend using only one inoculation site in the center of the petri plate. Multiple inoculations result in inhibition of twitching motility as the expanding colonies approach each other and this may skew results.

**13.** If necessary, plates can be stored at 4°C at this stage for measurement later. However, if you are doing any microscopy or image capture, this needs to be done immediately as the cold storage changes the interstitial colony morphology.

**14.** It is important to use an appropriate negative control especially if assaying clinical or mutant strains. Some strains may be capable of some, albeit, aberrant twitching motility and therefore show a reduced interstitial colony size. A non-twitching control will allow the assessment of the effects of growth and replication that result in a very small interstitial colony but are not attributable to twitching motility (see Fig. 1).

**15.** Do not leave in the developer solution for more than 30 minutes. If the solution is left on too long, you will scratch the surface of the agar in Step 22 and this may interfere with being able to see the white halo if it is small. Previous literature reports the use of Coomassie Brilliant Blue to stain the interstitial biofilm [4, 5]. This method involved dehydrating the medium with paper towel, staining and destaining the agar to visualise the interstitial colony. The method described here is simple, rapid, uses fewer consumables and visualises the interstitial colony without the need for dehydrating the medium.

**16.** It is important to visually inspect the boiling solution and to make sure that there are no small lumps in the solution. These will interfere with the gellan gum setting.

**17.** The TMGG solution can be used immediately (after cooling to 65°C) or it can be allowed to set, stored at room temperature, then melted later when required. When melting TMGG, use a microwave set to low power and monitor the solution to make sure it does not boil over. Make sure the solution is completely melted before using, as any small lumps will affect the ability to form flat surfaces for microscopy. Again take care when handling the heated solution.

**18.** Ensure the surface is level by using a spirit level. This is important or the slides will have an uneven surface and this makes microscopic inspection very difficult.

**19.** The slides may be stored for up to one week at 4°C before use. We recommend making all slides needed for one week from one batch of molten TMGG and discarding the disused portion.

**20.** Drying times will vary between different cabinets and will need to be optimised. 15 min drying is a good starting point, but it is useful to come to recognise how much drying a particular set of slides needs through tactile and visual senses. Slides that have been dried the right amount will have a slightly matt sheen over the surface of the TMGG and will be slightly tacky to handle.

**21.** If the cover slip is too warm, not only will the bacteria be burnt, moisture will be trapped due to condensation between the coverslip and the TMGG. It doesn't matter if there are a small number of bubbles trapped under the coverslip, as some will go away during incubation. You can use areas away from the bubbles for observation. The lowering of the coverslip step is the most difficult and you will get better with practice.

**22**. We recommend the use of high quality phase contrast microscopy rather than bright-field or differential interference contrast (DIC) microscopy to visualise individual cells.

## 5. References


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**6. Figure captions.**

**Figure 1.** Macroscopic Twitching Assay. The interstitial biofilm can be visualised by flooding the plates with TM developer solution and recording images of the petri plates on a flatbed document scanner. In these examples, wildtype *P. aeruginosa* strain PAK (left) had produced a large interstitial colony that has expanded due to twitching motility whilst the isogenic non-twitching *pilA* mutant (right) has produced only a small zone of growth. Panel A shows two petri plates that have been marked for measurement but are have not had TM developer solution added. Note that the top colony is clearly visible on both

plates and that the interstitial colony on the left plate is quite faint. Panel B shows two petri plates after TM developer solution has been applied. A 1 cm mark has been drawn onto one of the plates as a scale bar for quantification and to assist with accurate figure preparation where scale bars are required. Note that the top colonies have been removed and the interstitial colony on the left plate is more easily seen and the small interstitial colony seen on the right plate is attributable to growth only.

**Figure 2.** Microscopic Twitching Assay. Representative images of interstitial colonies formed by wildtype *P. aeruginosa* strain PAK visualised with phase contrast microscopy. Under the conditions of the assay twitching motility mediates active expansion of the interstitial colony and manifests distinct micro-morphological features including an intricate lattice-like network of cells that forms behind rafts of cells at the leading edge of the colony. Panels A and B show overviews of the outermost edge of the expanding interstitial colony obtained with 10x (A) and 20x (B) low magnification dry objectives. Panels C and D are high resolution images of the leading edge rafts (C) and lattice-like network (D) obtained with 100x oil immersion objective. Scale bars: panel A = 200 μm; panel B = 100 μm; and panels C and D = 20 μm.

**Figure 3.** Microscopic Twitching Assay. Prepare the microscope slides by flame sterilising and aligning side-by-side in a large petri plate. Push the slides together tightly until the long edge on one slide is firmly abutted to the rim of the

petri plate as shown in A. Pour the TMGG solution and tilt gently to ensure an even surface coverage, add the damp tissues and prepare for 4°C storage as shown in B.

**Figure 4.** Microscopic Twitching Assay. After slides are gently separated from one another, the slides are covered in an even layer of TMGG (A, upper slide). This is then trimmed as described in Step 22 to achieve a smaller rectangle of TMGG with clear edges and an area to label the slide (A, lower slide). After drying, the slide is inoculated and a glass coverslip gently lowered to create an interstitial interface (B).

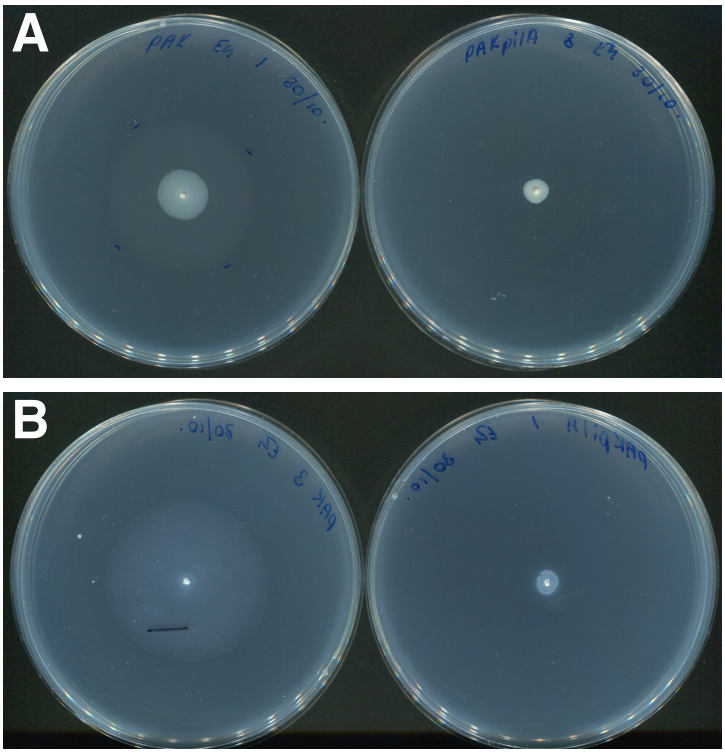


Figure 1

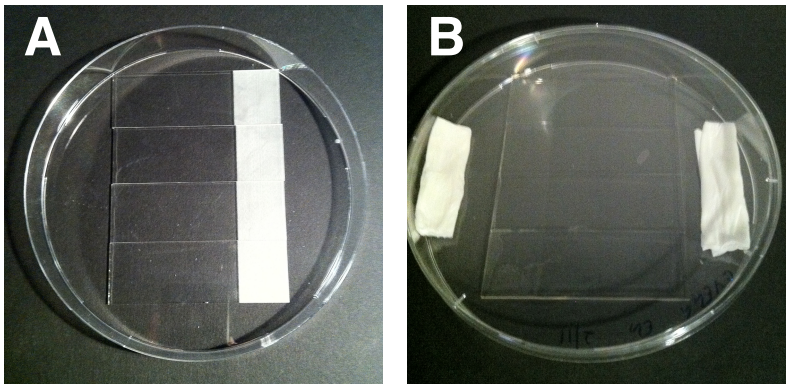


Figure 2

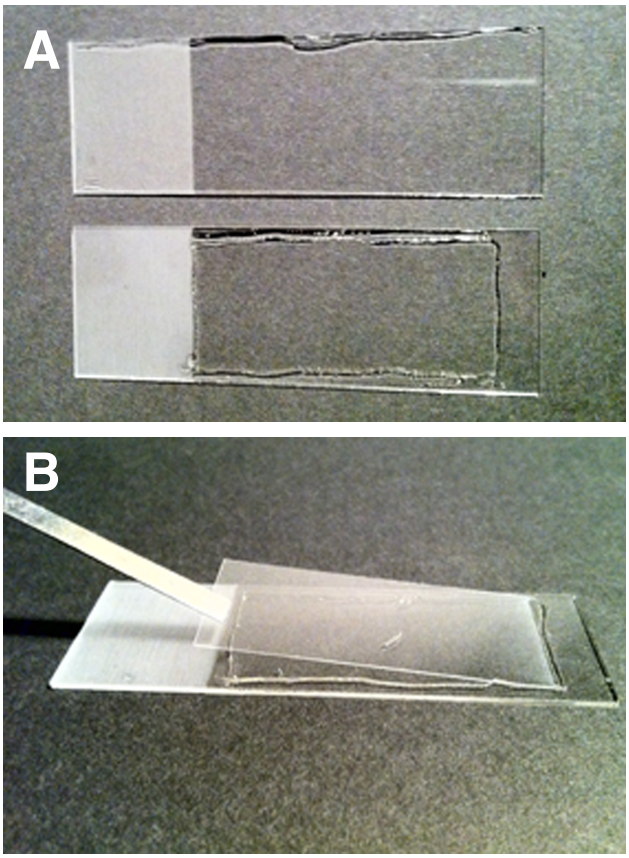


Figure 3

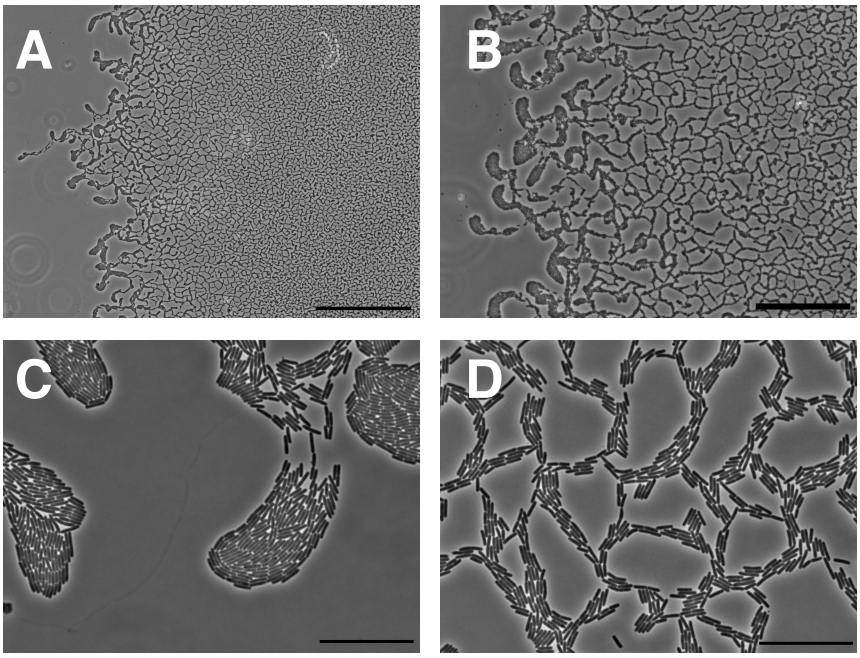


Figure 4