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1                   **Analysis of bacterial pilus assembly by shearing and**  
2                   **immunofluorescence microscopy**

3  
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9

10 **Abstract**

11  
12 Bacterial surface appendages of the type 4 pilus superfamily play diverse roles in adherence,  
13 aggregation, motility, signaling and macromolecular transport. Here we describe two  
14 analytical approaches to study assembly of type 4 pili and of pseudopili produced by type 2  
15 protein secretion systems: the shearing assay and immunofluorescence microscopy. These  
16 complementary antibody-based methods allow for semi-quantitative analysis of fiber  
17 assembly. The shearing assay can be scaled up to yield crude extracts of pili that can be  
18 further analyzed by electron and atomic force microscopy or by mass spectrometry.

19  
20 Running head: Pilus assembly assays  
21

22 **Key words:** type 4 pili, type 2 secretion pseudopili, pilus assembly, shearing assay, immuno-  
23 detection, Tricine SDS-PAGE, immunofluorescence microscopy  
24

## 1. Introduction

Type 4 pili (T4P) are thin filaments exposed on the surface of many bacterial and archaeal species [1]. They promote motility, adherence, cell signaling, biofilm formation, DNA uptake, or protein secretion [2]. T4P mediate colonization of tissues by promoting the surface adhesion and attachment, as well as inter-bacterial contacts leading to aggregation and micro-colony formation. These features make T4P key virulence factors of many human, animal and plant pathogens [2]. Many T4P are dynamic and able to retract upon binding to a surface, promoting a form of motility called twitching. Active pilus retraction generates remarkably high forces that pull the bacterial body towards the attachment point [3]. In some bacteria, T4P have been implicated in electron transport and conductance [4].

T4P biogenesis and dynamic function relies on a set of conserved proteins that are part of the superfamily known as type 4 filament (Tff) nanomachines [1]. All Tff systems assemble helical fibers from protein subunits embedded in the plasma membrane. Found in many bacterial species, T4P have common evolutionary origins with archaeal pili and flagella [5, 6]. Another prominent member of this superfamily is the bacterial type 2 protein secretion system (T2SS) [7, 8]. T2SSs assemble periplasmic fibers called pseudopili, which can be visualized on the bacterial surface upon overproduction of the T2SS assembly machinery and/or of the major pseudopilin subunit [9, 10].

The close evolutionary relationship between T4P and T2SS is illustrated by the ability of the *Klebsiella oxytoca* pullulanase T2SS to assemble fibers from T4P subunits [10, 11]. The same system can be used to assemble fibers from major pseudopilins cloned from a variety of heterologous T2SSs [12]. Here, we describe the use of the Pul T2SS machinery reconstituted in *E. coli* and cloned on a moderate copy-number plasmid to assemble filaments

51 from its cognate major pseudopilin subunit PulG, and from the *E. coli* T4P subunit PpdD. The  
52 same protocols can also be used to efficiently shear flagella or other types of pili (type 1 pili  
53 or pili from Gram-positive bacteria) from the bacterial surface, permitting downstream  
54 analyses in both the cell-bound and soluble fractions. Using antibodies specific for fiber  
55 subunits, this method allows for the global quantitative assessment of pilus assembly  
56 efficiency, expressed as a ratio between the assembled pilin subunits on the bacterial surface  
57 and the total amount of pilins, including both those assembled into bacterial cell-bound

58 fibers and the pool of pilin subunits in the plasma membrane awaiting assembly.  
59 Immunofluorescence microscopy is a complementary assay that provides insight into  
60 the fiber length and number of pili per cell, giving an estimate for the number of active  
61 assembly machineries. In contrast to the global approach, immunofluorescence  
62 microscopy only allows one to visualize fibers assembled on the cell surface. The  
63 complementarity of these two approaches is illustrated in previous studies of T2SS  
64 models [13, 14].

65

## 66 **2. Materials**

67

68 Standard analytical grade chemicals are purchased from established commercial  
69 suppliers. Prepare all solutions using ultrapure water.

70

71

72

73

74

75

76

### 2.1 Bacterial culture

1. *Escherichia coli* K-12 strain PAP7460 (MC4100  $\Delta(lac-argF)U169 araD139$   
*relA1 rps150  $\Delta malE444 malG501$  [F' *lacI<sup>Q</sup>Tn10*]) (see **Note 1**).*
2. Lysogeny Broth (LB).



77 Dissolve 10 g of bacto-tryptone, 5 g of yeast extract and 10 g of NaCl in 1 L of  
78 distilled H<sub>2</sub>O (dH<sub>2</sub>O). If required, adjust the pH to 7.0 with 1 N NaOH. Autoclave  
79 at 121°C for 20 min to sterilize.

80 3. Pilus inducing solid media: in this case we used LB with 1.5% agar sterilized  
81 as above and supplemented, after cooling to 45-50°C, with Ap (100 µg/mL),  
82 Cm (25 µg/mL) and 0.2% D-maltose.

83 4. An incubator, set to 30°C.

84

## 85 **2.2 Shearing of pili from the bacterial surface**

86

87 1. Sterile toothpicks or a platinum loop.

88 2. Vortex.

89 3. LB (around 3 mL per sample).

90 4. One 2-mL syringe with 26-Gauge needle for each sample.

91 5. A spectrophotometer at 600 nm and disposable 1-mL cuvettes.

92 6. A benchtop microcentrifuge (4°C).

93 7. 100% Trichloroacetic acid (TCA).

94 8. 100% acetone.

95 9. A ventilated fume hood.

96 10. SDS sample buffer (150 mM Tris-HCl (pH 6.8), 6% sodium dodecyl sulfate  
97 (SDS), 30% glycerol containing the tracking dye - typically bromophenol blue or  
98 phenol red at 0.05 mg/mL).

99

## 100 **2.3 Tris-Tricine denaturing gel electrophoresis**

101

- 102 1. A vertical gel caster system and slab gel electrophoresis apparatus with a DC  
103 power supply.
- 104 2. 70 % ethanol (v/v).
- 105 3. 40 % acrylamide-bis solution (37.5:1), stored at 4°C.
- 106 4. Tricine gel buffer (3 x): 3 M Tris, 1 M HCl, 0.3 % (w/v) SDS, pH 8.45.
- 107 5. 10% (w/v) ammonium persulfate (APS), stored in aliquots at -20°C.
- 108 6. N, N, N, N'-Tetramethyl-ethylenediamine (TEMED), stored at room temperature.
- 109 7. Anode buffer 10 x (for bottom reservoir): 1 M Tris, 0.225 M HCl, pH 8.9.
- 110 8. Cathode buffer 10 x (for top reservoir): 1 M Tris, 1 M Tricine, 1 % (w/v) SDS, pH  
111 8.25 (if made correctly, pH does not need to be adjusted).

112

## 113 **2.4 Immunoblotting**

114

- 115 1. Nitrocellulose membranes (0.45 µm pore) optimized for ECL.
- 116 2. Whatman 3MM chromatography paper.
- 117 3. Millipore tweezers.
- 118 4. A fast blotter (Pierce G2, Thermo Fisher Scientific) or any other semi-dry electro-  
119 transfer apparatus.
- 120 5. 1-Step Transfer Buffer (Thermo Fisher Scientific) or standard Transfer buffer (25  
121 mM Tris-HCl, 192 mM glycine, 10% ethanol, pH 8.3).
- 122 6. 0.2 % Ponceau S (w/v) solution in 3 % TCA. This solution is stored at room  
123 temperature and reused.
- 124 7. Tris-buffered saline with Tween-20 (TBST): 50 mM Tris, 150 mM NaCl, 0.05%  
125 Tween-20, pH 7.6.

- 126 8. Blocking solution: 5% non-fat milk in TBST or 1% BSA in TBST.
- 127 9. Primary antibody to detect pilin subunit, appropriately diluted in blocking solution
- 128 (see **Note 2**).
- 129 10. Secondary antibody: horseradish peroxidase-coupled anti-rabbit antibody, diluted
- 130 1:40 000 in TBST.
- 131 11. Enhanced chemiluminescence Pierce ECL2 western blotting substrate.
- 132 12. A chemiluminescence detection apparatus.

133

## 134 **2.5 Immuno-fluorescence microscopy**

135

- 136 1. A portable vacuum aspiration system that can be placed under the fume hood.
- 137 2. An ultrasonicator (with standard small probe).
- 138 3. Delicate task wipers.
- 139 4. Clear nail polish.
- 140 5. Dulbecco's phosphate buffered saline (PBS) without MgCl<sub>2</sub> or CaCl<sub>2</sub>.
- 141 6. Blocking solution: 1% BSA in PBS.
- 142 7. Poly-L-lysine hydrobromide stock solution (1 mg/mL in dH<sub>2</sub>O) filter sterilized, store
- 143 at -20°C. Poly-L-lysine working solution (100 µg/mL). Prepare by diluting stock
- 144 solution 1:10 with PBS.
- 145 8. Cleaned rectangular coverslips 22 mm x 22 mm. An ultrasonication protocol is used
- 146 to clean the coverslips, as described in section (3.4).
- 147 9. Microscope slides (75 mm x 26 mm).
- 148 10. Tissue culture test plates with 6 wells.

- 149 11. 37 % paraformaldehyde (PFA) stock solution in dH<sub>2</sub>O (pH 7.4), filter sterilize and  
150 store at -20°C. Prepare working solution by diluting stock solution 1:10 in PBS, filter  
151 sterilize and store at -20°C (*see Note 3*).
- 152 12. 1 M Tris-HCl pH 8.0.
- 153 13. Primary antibody of interest; for this protocol polyclonal rabbit antisera raised against  
154 pilus subunits were diluted 1:1000 in 1% BSA-PBS.
- 155 14. Secondary antibody; Alexa 488-coupled goat anti-rabbit IgG diluted to 1:200 in 1%  
156 BSA-PBS.
- 157 15. ProLong Gold Antifade Reagent with DAPI.
- 158 16. Microscopy tweezers with fine tips.
- 159 17. A fluorescence microscope equipped with a digital camera.
- 160 18. Immersion oil.

161

## 162 **3. Methods**

163

### 164 **3.1 Shearing of pili from the cell surface**

165

- 166 1. Streak a dense bacterial lawn on pilus-inducing agar media, incubate at 30°C for 48  
167 hrs.
- 168 2. Scrape the bacteria off the plates with a sterile toothpick or sterile platinum loop and  
169 transfer into a microcentrifuge tube containing 1 mL of LB. Resuspend by pipetting  
170 until clumps are dissolved (*see Note 4*).
- 171 3. Measure the OD<sub>600nm</sub> using 1 mL of LB as a blank. Dilute the sample, if necessary, to  
172 ensure the measurement is within the linear range of the spectrophotometer.
- 173 4. Normalize all samples to 1 mL, OD<sub>600nm</sub> = 1.
- 174 5. Vortex suspensions continuously for 2 min at maximum speed.

- 175 6. Pass suspensions through a 26-gauge needle 6 times to shear pili from the cell surface  
176 (see **Note 5**).
- 177 7. Centrifuge the suspension 15 min at 16,000 x g in a microcentrifuge at 4°C.  
178 Transfer 0.85 mL of supernatant (SF 1) from the topmost fraction to a clean microfuge  
179 tube, while avoiding disturbing the pellet. Discard the remaining supernatant (0.15  
180 mL) from the cell fraction (CF) and resuspend the pellet in 100 µL of SDS sample  
181 buffer. Reserve the CF sample on ice or at -20°C for SDS-PAGE analysis.
- 182 8. Centrifuge the tube containing 0.85 mL of SF 1 for 10 min, at 16,000 x g at 4°C. This  
183 step will remove remaining bacteria from the supernatant.
- 184 9. On ice, transfer 0.7 mL of supernatant off the topmost fraction into a clean  
185 microcentrifuge tube, now called sheared fraction 2 (SF 2) (Fig. 1).
- 186 10. Place the tube on ice. Add 1/10th volume (70 µL) of cold 100% TCA solution to  
187 obtain a final concentration of 10% TCA. Vortex immediately (see **Note 6**).
- 188 11. Incubate the solution on ice for 30 min.
- 189 12. Place the tubes in the same orientation in the microcentrifuge and pellet at 16,000 x g  
190 for 30 min to 1 hr at 4°C.
- 191 13. Carefully aspirate the supernatant (see **Note 7**).
- 192 14. Add 1 mL of cold acetone (kept at -20°C) to wash the pellet, without resuspending.  
193 Centrifuge at 4°C for 2 min at 16,000 x g. Aspirate off the supernatant carefully.
- 194 15. Repeat acetone wash as above. Air-dry the pellet while keeping the eppendorf tubes  
195 open on ice under the fume hood for 10-15 min. Resuspend in 70 µL of SDS sample  
196 buffer.
- 197 16. Pilins are relatively abundant proteins. Analyze equivalent amounts of sample  
198 (typically to 0.05 OD<sub>600nm</sub> for CF and SF) by Tris Tricine SDS-PAGE as described in  
199 Section 3.2. (Fig. 2). This electrophoresis system allows small proteins to be well

200 resolved, rendering it highly suitable for pilins that have a size range from 10 to 20  
201 kDa.

202

### 203 **3.2 Tris-Tricine SDS-PAGE**

204 Analysis of concentrated cell and sheared fractions on 10 % Tris Tricine SDS-PAGE [15] (*see*  
205 **Note 8**).

206 1. Wipe gel plates with 70% ethanol and cast the gel (any vertical slab gel apparatus is  
207 suitable).

208 2. Prepare 15 mL of the separating gel solution (enough for 4 BioRad mini Protean II gels  
209 or two Apelex Mini wide Vertigel 2 gels) by mixing 6.25 mL dH<sub>2</sub>O, 5 mL 3 x Gel buffer  
210 and 3.75 mL acrylamide solution. Add 75 μL 10 % APS and 30 μL TEMED, mix well  
211 and pour immediately between the glass plates leaving space for the stacking gel. Overlay  
212 gently with 1 mL dH<sub>2</sub>O and allow to polymerize at room temperature. Once polymerized,  
213 pour out dH<sub>2</sub>O and carefully wick out any remaining dH<sub>2</sub>O using a piece of Whatman  
214 3MM chromatography paper.

215 3. Clean the combs with 70 % ethanol and prepare the stacking gel solution by combining  
216 5.7 mL dH<sub>2</sub>O, with 3.3 mL 3 x gel buffer and 1 mL acrylamide. Add 90 μL 10 % APS  
217 and 40 μL TEMED, mix well and pour on top of separating gel. Insert the comb  
218 immediately and leave at room temperature until polymerized.

219 4. Place the gel into the apparatus. Fill the top tank with 1 x Cathode buffer and the bottom  
220 with 1 x Anode buffer. Remove combs and wash the wells by pipetting gently.

221 5. Load a well with 2 μL pre-stained molecular weight marker and the equivalent of 0.05 to  
222 0.1 OD<sub>600nm</sub> of each CF and SF. Run the gel at a constant current of 30 mA per Biorad  
223 minigel (typically 2 h 30 min), until the tracking dye reaches the bottom of the gel.

224

225 **3.3 Western Blot and Immuno-detection**

226

- 227 1. Remove the gel from the apparatus, separate the glass plates and use a sharp tool to  
228 remove the stacking gel. Transfer the separating gel into a container containing 10 mL  
229 of Transfer buffer and equilibrate 5 min with gentle agitation on a benchtop shaker.
- 230 2. Set up the electro-transfer: Prepare 10 sheets of Whatman 3MM paper and cut the  
231 nitrocellulose membrane slightly larger than the size of the gel. Wet the anode  
232 (bottom) plate of the transfer apparatus with 3 mL transfer buffer. Soak the Whatman  
233 paper in transfer buffer and stack five sheets on the anode plate; use a plastic roller or  
234 tube to remove air bubbles between each layer. Wet the nitrocellulose membrane and  
235 place it on the stack, center the gel on the membrane and cover with the remaining 5  
236 sheets of presoaked Whatman paper. Wet the cathode (top plate), place it on top of the  
237 stack, press with even pressure to seal the plates together and insert the closed cassette  
238 into the transfer apparatus.
- 239 3. Transfer proteins from the gel onto the membrane using the standard preset program  
240 for mixed molecular weight range (at 25V and 1.3 A, for 7 min). Alternatively,  
241 standard transfer buffer and a semi-dry transfer apparatus can be used in this step; in  
242 that case transfer should proceed for 45 min at a constant current of 100 mA.
- 243 4. Remove the membrane from the transfer apparatus and stain with Ponceau S solution  
244 for 5 min. Destain with dH<sub>2</sub>O using a squirt bottle, until protein bands appear, verify  
245 even transfer and take a picture of the result.
- 246 5. Wash the membrane in 20 mL TBST with gentle agitation, until the red protein bands  
247 are no longer visible. Discard the destaining solution.
- 248 6. Cover the membrane in blocking solution and incubate for 1 hr at room temperature  
249 with mild agitation on a benchtop shaker. Discard the blocking solution.

- 250 7. Add the primary antibody working solution and incubate for 1 hr with mild agitation  
251 as above, at room temperature. Remove primary antibody. If desired, primary  
252 antibody solution can be stored at -20°C and reused 5-10 times.
- 253 8. Wash the membrane four times for 10 min in TBST.
- 254 9. Add the secondary antibody working solution and incubate for 1 hr with agitation.  
255 Discard the secondary antibody solution.
- 256 10. Wash the membrane four times for 10 min in TBST.
- 257 11. Blot excess liquid from the membrane using a sheet of Whatman filter paper. Prepare  
258 the ECL2 developing solution by mixing 2 mL of solution A with 50 µL of solution B  
259 in a dish made of glass or inert plastic (polyallomer). Soak the membrane face down  
260 in this solution for 5 min to activate the chemiluminescent reaction.
- 261 12. Record the signal (emitted light) using the chemiluminescence imager.  
262 Quantify the signal for the mature pilin in cell- and sheared fractions using Image J.  
263 Piliation efficiency is expressed as the percentage of total signal present in the sheared  
264 fraction, i.e.:  $E = d_{SF} / (d_{SF} + d_{CF}) \times 100$ . E represents piliation efficiency (in %) and d  
265 represents the measured density of sheared fraction (SF) and cell fraction (CF)  
266 expressed in arbitrary units.

267

### 268 **3.4 Ultrasonication of coverslips for immunofluorescence microscopy**

269 (*see Note 9*).

- 270 1. Place the coverslips on a rack inside a wide crystallization beaker and add dH<sub>2</sub>O, so  
271 that the rack is fully immersed in liquid.
- 272 2. Place the ultrasonicator tip in the liquid close to the rack without touching it.
- 273 3. Ultrasonicate at 23-27% for 20 min using 30 s pulse and 30 s pause intervals.
- 274 4. Wash the rack twice with sterile dH<sub>2</sub>O.



- 275 5. Wash the rack with 90% ethanol.
- 276 6. Wash 3 times with sterile dH<sub>2</sub>O.
- 277 7. Aspirate the excess of water from in between coverslips. Cover the beaker containing
- 278 the coverslip rack with aluminum foil and dry at 42°C overnight. The coverslips can be
- 279 prepared days in advance and stored at room temperature protected from dust with
- 280 parafilm and aluminum foil.

281

### 282 **3. 5. Immunofluorescence microscopy**

283

- 284 1. Place one clean coverslip per well in the 6-well tissue culture test plate. Coat the surface of
- 285 each coverslip with 1 mL of the poly-L-Lysine working solution, ensuring the entire
- 286 surface is covered. Close the lid of the tissue culture test plate and incubate at 37°C for 1
- 287 hr.
- 288 2. Aspirate the liquid using a vacuum pump and dry at 37°C (*see Note 10*).
- 289 3. Wash the coverslips 3 times with dH<sub>2</sub>O and dry at 37°C.
- 290 4. The bacterial samples for the immunofluorescence microscopy assay are grown using the
- 291 same pilus-inducing conditions described for the shearing assay (section 3.1). For
- 292 immunofluorescence microscopy assay, bacteria are removed from the plate using a sterile
- 293 inoculation loop and transferred into 1 mL PBS. Gently rotate the loop to resuspend the
- 294 cells without shearing the pili (*see Note 11*).
- 295 5. Measure the OD<sub>600nm</sub> using 1 mL PBS as a blank. Dilute the sample, if necessary to ensure
- 296 the measurement is within the linear range of the spectrophotometer.
- 297 6. Normalize all samples to 1 mL, 0.1 OD<sub>600nm</sub> (*see Note 12*).
- 298 7. Cover the entire surface of the coverslip with the bacterial suspension (*see Note 13*).
- 299 8. Incubate the culture plate at room temperature, static for 1 hr.

- 300 9. Wash 3 times with 1 mL PBS gently by adding and aspirating the liquid on the side.  
301 Avoid touching or disturbing the surface of the coverslip.
- 302 10. In a fume hood, add 1 mL of 3.7 % PFA in PBS working solution and incubate the samples  
303 for 30 min at room temperature in the fume hood to fix the bacteria and pili to the  
304 coverslip.
- 305 11. In a fume hood, stop the reaction by adding 500  $\mu$ L 1 M Tris-HCl (pH 8.0). Remove the  
306 liquid immediately and dispose in the appropriate toxic liquid waste container.
- 307 12. Add 1 mL 1 M Tris-HCl (pH 8.0) and incubate 5 min at room temperature.
- 308 13. Wash 3 times with 1 mL PBS.
- 309 14. Flood the surface of the coverslip with blocking solution (1 % BSA in PBS). Incubate 1 hr  
310 at room temperature without shaking. Use vacuum to aspirate blocking solution.
- 311 15. Cover the entire surface of the coverslip with primary antibody solution (500  $\mu$ L), incubate  
312 1 hr at room temperature without shaking. Aspirate off the primary antibody solution.
- 313 16. Wash the coverslip by flooding the coverslip with 1 mL PBS, gently adding PBS to the  
314 space next to the coverslip until the coverslip is fully covered with PBS. Incubate for 1 min  
315 without shaking and then aspirate all liquid. Repeat this step two more times.
- 316 17. Cover the entire surface of the coverslip with secondary antibody solution (500  $\mu$ L of  
317 fluorophore-coupled anti-rabbit diluted in PBS), protect the samples from light and  
318 incubate 1 hr at room temperature without shaking (*see Note 14*). Aspirate off the liquid.
- 319 18. Wash 3 times with 1 mL PBS as in Step 16.
- 320 19. Place the microscope slides on a piece of aluminum foil. Clean with tissue paper and 70 %  
321 ethanol. Air dry.
- 322 20. Put a 10- $\mu$ L drop of the ProLong Gold Antifade Reagent with DAPI on the microscope  
323 slide to stain the bacterial nucleoid (*see Note 15*).

- 324 21. With microscopy tweezers (with the aid of a needle if necessary), carefully remove the  
325 coverslip from the tissue culture test plate and use tissue paper to wick off excess liquid  
326 from the edge of the coverslip, be sure not to touch the fixed sample. Place the coverslip  
327 face down in the center of the DAPI-ProLong drop. Press down gently to remove air  
328 bubbles.
- 329 22. Apply a spot of nail polish to the corners of the coverslip. Let dry in the dark.
- 330 23. Apply nail polish around the edges of the coverslip to seal, let dry in the dark and store the  
331 slides in the dark at room temperature.
- 332 24. Samples are examined using a fluorescence microscope equipped with 63 x or 100 x  
333 immersion oil objectives, using blue (DAPI) and green (Alexa 488) filters. Here, samples  
334 were visualized with an upright Axio Imager A2 microscope (Zeiss), images were captured  
335 using the AxioCam MRm digital camera connected to the microscope. Images were  
336 analyzed with Zen 2012 software (Fig. 3) (*see Note 16*).

337

#### 338 4. Notes

- 339 1. For pilus assembly assays PAP7460 was co-transformed with plasmid pCHAP8185  
340 (Ap<sup>R</sup>) encoding the complete set of *pul* genes from the *Klebsiella oxytoca* T2SS, or its  
341 derivatives harboring gene-deletions and a compatible empty vector pSU18/pSU19 (Cm<sup>R</sup>)  
342 or its derivatives encoding major T4 pilin *ppdD* or major T2SS pseudopilin *pulG*. To  
343 induce the *pul* genes encoding the fiber assembly machinery, 0.2 % D-maltose is added to  
344 LB Ap Cm plates. Depending on the expression system different antibiotics and/or  
345 inducing media may be necessary.
- 346 2. Primary antibody is diluted to its working concentration in TBST-5% milk. Here,  
347 custom rabbit polyclonal antibodies were raised against Male-PpdD, PulGsp-His and  
348 LamB were diluted to 1:1000, 1:2000 and 1:1000, respectively. To reduce the number of

349 non-specific proteins visualized during immuno-detection, the primary polyclonal antisera  
350 can be purified by adsorbing against a bacterial extract lacking the antigen of interest.  
351 Here, bacterial extracts were produced by growing the appropriate PAP7460 strain lacking  
352 the antigen of interest under *pul* inducing conditions, cells were collected by  
353 centrifugation, resuspended in PBS and broken open using a cell disrupter, resulting cell  
354 extracts were then stored at -20°C until used. To adsorb the antibodies, bacterial extracts (1  
355 mL) were combined with undiluted primary serum (40 µL) in 1.5-mL Eppendorf tubes and  
356 incubated for 1 hr at 37°C. Antigen-antibody complexes and debris were removed by  
357 centrifugation at 16,000 x g for 5 min at 4°C. Clarified supernatants were transferred to a  
358 fresh tube and were diluted in TBST or PBST to the appropriate working concentration.

359 3. PFA is classified as a toxic, inflammable, harmful product, security measures must be  
360 taken for the preparation of the solution; weigh and dissolve the compound in a chemical  
361 hood, wear appropriate personal protective equipment such as gloves, goggles and a mask  
362 equipped with a particle filter. PFA is soluble at alkaline pH. To prepare the stock solution  
363 add 500 µL of 7.5N NaOH to 100 mL of PFA in dH<sub>2</sub>O and heat to 55°C to dissolve. Do  
364 not overheat as it can denature the PFA. When fully dissolved, adjust the pH back to 7.4  
365 with concentrated HCl. Filter and store frozen in aliquots.

366 4. After this point, maintain samples on ice or at 4°C.

367 5. Passing samples through the 26 G needle is not required to shear PulG pseudopili,  
368 however, this step significantly improves the yield of PpdD T4P. Whether or not this step  
369 is required depends on the thickness and elasticity of the surface fibers and should be  
370 determined empirically for each system.

371 6. Addition of a carrier protein, such as BSA (final concentration 1 µg/mL) can improve  
372 TCA precipitation efficiency. Carrier proteins can also serve as useful loading and transfer  
373 controls after protein electro-transfer and can be tracked by Ponceau S staining.

374 7. At this step, the TCA precipitated protein pellet may be invisible or may detach from the  
375 bottom of the tube; aspirate off the supernatant very carefully using a drawn-out Pasteur  
376 pipet or a micro-tip from the opposite side of where the pellet is expected to accumulate.

377 8. Acrylamide is a neurotoxin, wear appropriate personal protective equipment when  
378 handling, such as gloves and goggles. Discard contaminated waste in appropriate  
379 containers.

380 9. Ideally, this step should be performed in a closed chamber, devoid of personnel. Any  
381 person present in the room during ultrasonication must wear hearing protection ( earmuffs).

382 10. At each drying step, it is important for the coverslip to be completely dry before  
383 proceeding to the next step.

384 11. It is critical to be very gentle at this step; too much movement will detach pili from the  
385 cell surface.

386 12. The amount of the sample analyzed will depend on the plasmid copy number; in this  
387 case we use 0.05 OD<sub>600nm</sub> for a medium- and 0.1 OD<sub>600nm</sub> for low copy-number plasmids.

388 13. Use wide-mouth pipette tips (cut the tip off with sterile scissors) when transferring  
389 bacterial suspensions to reduce shearing.

390 14. To prevent photo-bleaching, it is critical to protect samples from light during this step.  
391 Covering the tissue culture test plate with aluminum foil, placing samples in a dark room,  
392 or a closed drawer will protect samples from light.

393 15. As in Note 13, use wide mouth-pipette tips to transfer the ProLong reagent to the  
394 microscopy slide. ProLong is an anti-fade reagent that attenuates photo-bleaching during  
395 fluorescence imaging; the ProLong reagent used here contains DAPI DNA stain. Two  
396 coverslips can be placed on one microscopy slide.

397 16. The numbers and lengths of pili can be quantified in a representative number of fields  
398 and the data can be analyzed with appropriate methods to compare the piliation phenotypes

399 of different strains grown under the same conditions. Examples of such semi-quantitative  
400 analysis is provided in [13, 14].

401

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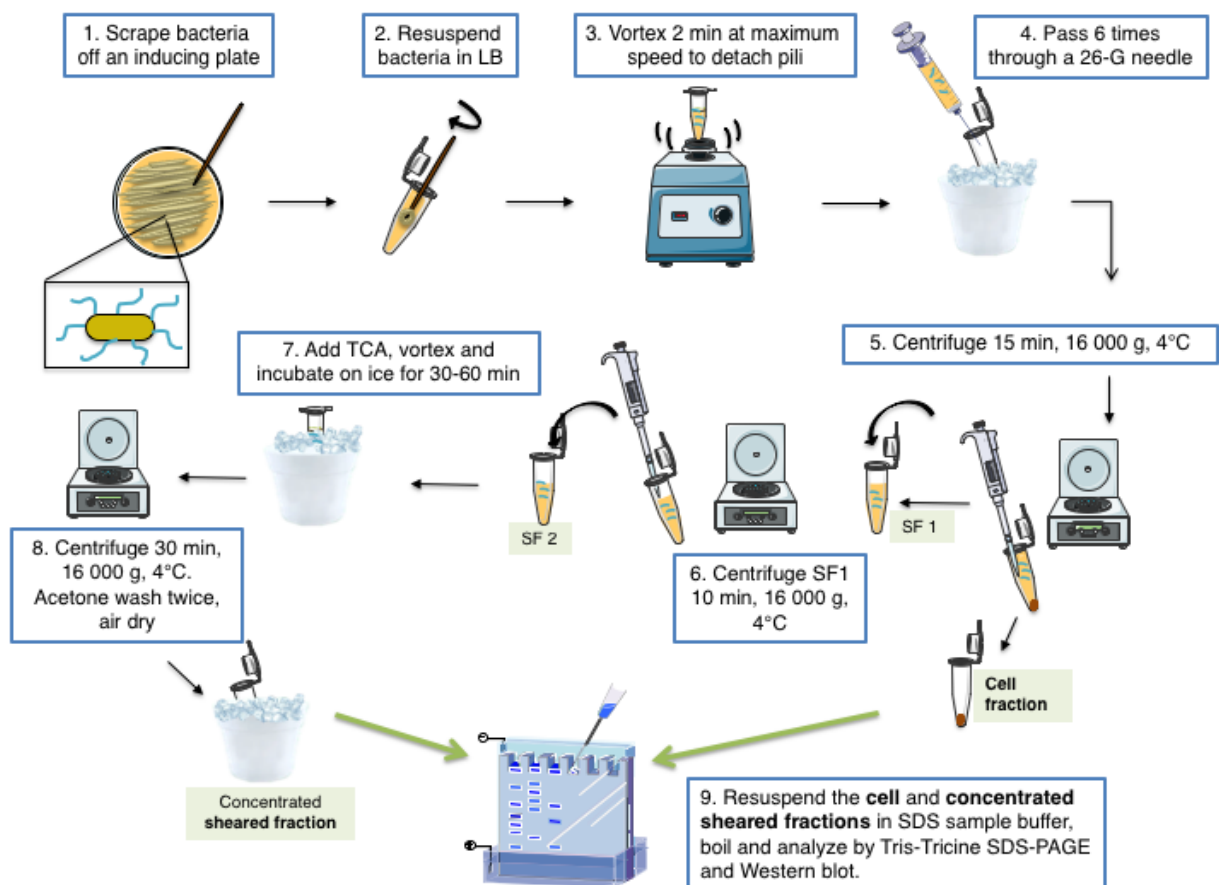
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457 **Figures and legends.**



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459 **Figure 1.** Shearing assay workflow. Shown is a schematic of the workflow for a shearing  
460 assay, with individual steps numbered and indicated in boxes.

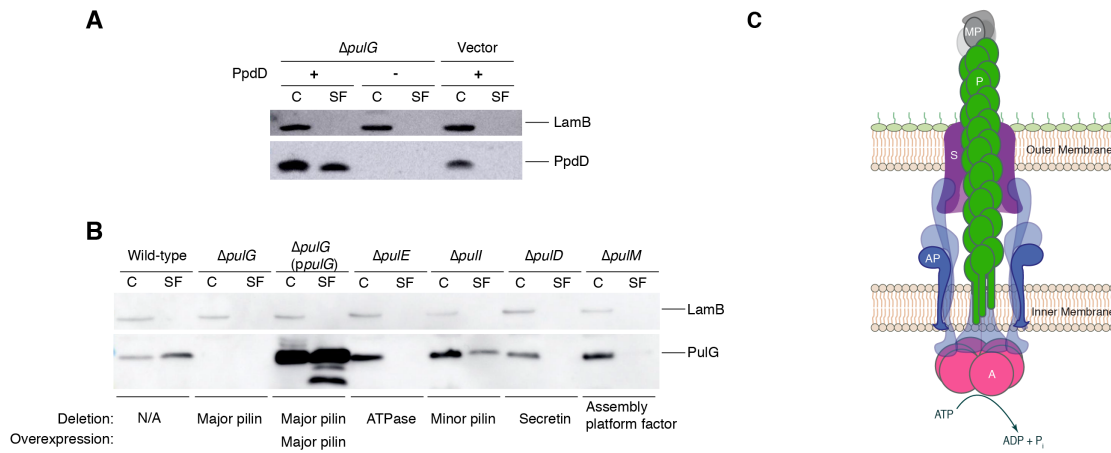
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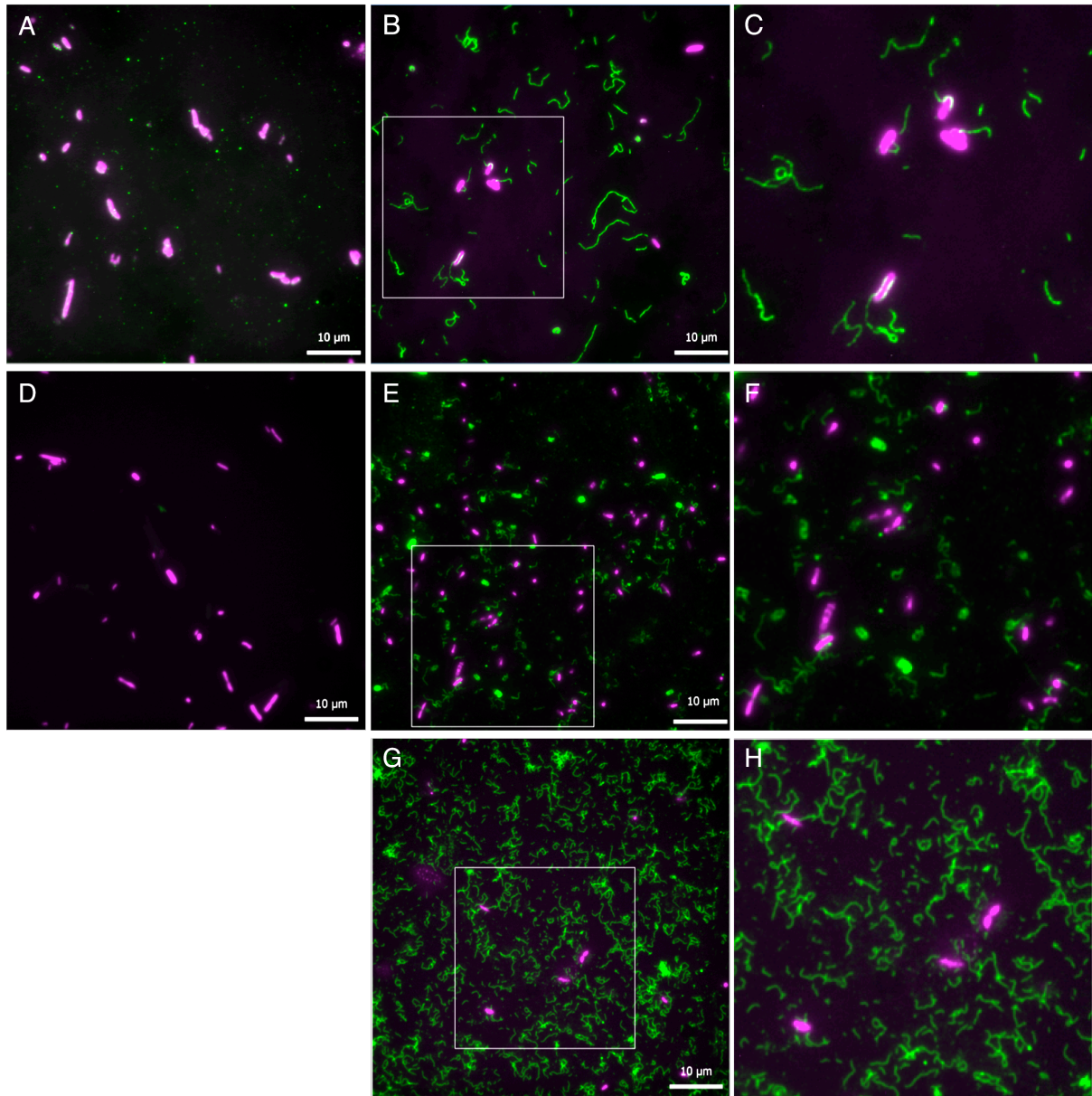
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**Figure 2.** Assembly of the PpdD and PulG pili by the Pul T2SS in *E. coli* K-12. (A) Immuno-detection of PpdD in cell fractions (C) and sheared fractions (SF) of *E. coli* K-12 strain PAP7460 co-expressing the Pul system and/or PpdD pilin indicated by (+), or co-expressed with the empty vector in each case indicated by (-) as negative controls. (A and B) The outer membrane protein LamB was used as an indicator for outer membrane contamination in the sheared fraction. Other abundant outer membrane markers can also be used. (B) Immuno-detection of PulG in cell fractions (C) or sheared fractions (SF) of *E. coli* K-12 strain PAP7460 co-expressing the Pul system (Wild-type), or its derivatives harboring gene-deletions (indicated) and a compatible empty vector pSU19 or its derivatives encoding major T2SS pseudopilin *pulG*. When applicable, the T2SS component missing due to gene deletion or overexpression of a component is indicated underneath the immunoblot. (C) Schematic of an assembled T2SS/T4P machine. Missing components from panel B: major pilin (P), ATPase (A), minor pilin (MP), secretin (S) and assembly platform (AP) factor proteins are indicated.



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484

485 **Figure 3.** Immunofluorescence microscopy analysis of PpdD (A-C) and PulG (D-H) fiber  
486 assembly. DAPI staining was used to detect DNA in fixed bacteria (magenta) and surface  
487 fibers were detected using PpdD (A- C) or PulG (D-H) antibodies (green). (A) and (D),  
488 Negative control cells expressing the Pul T2SS without the major pilin subunit from plasmid  
489 pCHAP8184 ( $\Delta pulG$ ) and empty vector; (B) Cells co-expressing pCHAP8184 and the T4P  
490 subunit encoded by the *ppdD* gene (pCHAP8565) [13]. (C) Enlarged area of interest  
491 highlighted in panel B. (E) Bacteria expressing the complete set of *pul* genes from plasmid  
492 pCHAP8185 and empty vector. (F) Enlarged area of interest highlighted in panel E. (G)  
493 Bacteria co-expressing pCHAP8184 ( $\Delta pulG$ ) and the T2SS major subunit encoded by the  
494 *pulG* gene (pCHAP8658) [14].  
495