Published in final edited form as:

Methods Mol Biol. 2017 ; 1615: 311–320. doi:10.1007/978-1-4939-7033-9_25.

Shearing and enrichment of extracellular Type IV pili

Alba Katiria Gonzalez Rivera and Katrina T. Forest*

Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706

Summary

Pili are wide-spread among bacteria. Type IVa pili (T4aP) are associated with a variety of bacterial functions including adhesion, motility, natural transformation, biofilm formation, and force-dependent signaling. In pathogenic bacteria, T4aP play a crucial role during infection and have been the target of hundreds of studies. Methods for isolation and purification of T4aP were first described in the 1970s. Purified pili have been used for studies of filament protein content, morphology, immunogenicity, post-translational modifications, and x-ray crystallography. We detail a tried-and-true method for isolation of large amounts of native T4aP from bacterial surfaces. The method requires only standard supplies and equipment available in most microbiology labs.

Keywords

T4P; fimbriae; pili; filament; shear; isolation

1. Introduction

Pili are hair-like appendages displayed by many pathogenic and environmental bacteria. One well-studied class is the Type IV pili (T4P). At approximately 6 nm in diameter, T4P filaments are thinner than flagella, and can reach many micrometers in length (1). They are expressed in some species at cell poles (*e.g.* in *Myxococcus xanthus* or *Pseudomonas aeruginosa*) (2, 3) and in others peritrichously (*e.g.* in *Neisseria gonorrhoeae* or *Deinococcus geothermalis*) (4, 5). Found in diverse Gram negative and Gram positive species (6, 7), T4P are involved in various bacterial functions including adherence, microcolony formation, biofilm initiation, and long-range electron transfer (8). One distinctive feature of these filaments is the capacity of some to retract, a property which has been associated with phage sensitivity, force generation, twitching motility, natural transformation and virulence (8).

T4P from Gram-negative organisms can be subdivided into two families; subtype a (T4aP) and subtype b (T4bP), with notable differences both in gene organization and biochemical properties. At the genome level, genes for T4aP subunits and assembly machinery are located in multiple operons dispersed around the chromosome, whereas those for T4bP are found to cluster in a single genomic region (9). We describe a method for the isolation of T4aP that capitalizes on their properties in two kinds of buffers (10). T4aP filaments can be

*For correspondence: forest@bact.wisc.edu.

isolated by disaggregation in high-pH, low salt buffer and aggregation (due to bundling) in buffer with near neutral pH and physiological salt concentration. Cycles of these two steps can increase the purity of a preparation, as contaminants that don't share these solubility properties are differentially lost. This method is not ideal for the more hydrophobic T4bP, which in some cases are successfully purified using an ammonium sulfate precipitation procedure (11).

Depending on the application, pili may be further purified. For example, for generation of anti-pilus antibodies, it may be desirable to remove LPS. This can be accomplished by incubation of the purified pili with polymixin B agarose. For high-resolution crystallographic studies of the full-length pilin monomer, dissociation of filaments in nondenaturing detergent followed by filtration is appropriate (12–15).

2. Materials

2.1. Growth and harvest of pilated bacteria

1. Bacterial strain: *P. aeruginosa* PAK/2Pfs or PAK *pilT* (see Notes 1 and 2)
2. 60 Tryptic Soy Agar (TSA) plates, 1.5% agar (see Notes 3 and 4)
3. Tryptic Soy Broth (TSB) (see Note 5)
4. 30°C incubator (see Note 6)
5. Stereo microscope
6. Glass Spreader
7. Inoculating turntable
8. 50 mL disposable conical tube
9. 70 % v/v Ethanol Solution
10. Bunsen burner

2.2. Collection of pili

1. High-speed floor model centrifuge and appropriate rotor (e.g. Beckman JA25.50)

¹*P. aeruginosa* must be handled in a Bio-safety Level 2 Laboratory. Follow Bio-safety procedures. These procedures will produce aerosols that could contain the pathogen. This method will yield high amounts of biohazardous waste. All steps here should be carried out under BSL-2 conditions with appropriate personal protection and caution against aerosol release of bacteria.

²PAK/2Pfs is a strain with nonretractile pili (17) caused by a mutation in the *pilT* gene (18). PAK/2Pfs is available from ATCC as strain 53308, and is also known as PAK2.2 (19). Strains that lack the PilT pilus retraction motor express higher amounts of pili than retraction proficient strains and thus are commonly used for pilus purification with yields up to 10 times higher than wild type strains (17, 20). We have achieved similar results with a PAK derivative carrying an in-frame deletion in the *pilT* gene (a gift of Dr. Stephen Lory, Harvard Medical School). In this chapter data are presented for the PAK *pilT* strain.

³Prepare TSA following manufacturer's instructions or by mixing 15 g of tryptone, 5 g of soytone, 5 g of NaCl and 15 g of agar per liter of purified water. Autoclave to sterilize. Pour 20–22 mL into 100 × 15 mm Petri dishes. Allow the plates to dry (with lids on) at room temperature for 2–3 days.

⁴In our experiments, using TSA or LB in 1.5 % agar plates yields equivalent amounts of pili based on the assessment by SDS-PAGE.

⁵Prepare TSB media as directed by manufacturer or dissolve and sterilize 17 g of tryptone, 3 g of soytone, 2.5 g of D-glucose, 5.0 g of NaCl, and 2.5 g of di potassium hydrogen phosphate per liter of purified water.

⁶Avoid incubating the plates in high humidity. If applicable leave out water tray.

2. Oak Ridge centrifuge tubes with O-ring screw caps (Maximum RCF > 17,500 × g). These are needed due to the handling of a BSL-2 organism.
3. 250-mL beaker
4. Magnetic stir bar and magnetic stir plate
5. Parafilm
6. 25 mL and 5 mL serological pipets
7. Plastic transfer pipets
8. Pilus Disaggregation Buffer (PDB): 1 mM DTT, 150 mM ethanolamine, pH 10.5 (4°C). DTT must be added shortly before use.

2.3. Purification of pili

1. Centrifuge tubes (Maximum RCF > 20,000 × g). 50 mL disposable conical centrifuge tubes are convenient since at this stage, BSL-2 safety containment is not required at this stage. Standard Oak Ridge tubes are fine too.
2. Pilus Bundling Buffer (PBB): 150 mM NaCl, 0.02 % NaN₃, 50 mM Tris-HCl, pH 7.5 (4°C)
3. Dialysis tank or large flask (at least 4 L)
4. Dialysis tubing (MWCO > 3.5 kDa, 29 mm diameter)

2.4. Assessment of results

1. 16 % Tricine-SDS-PAGE gel (*see* Note 7)

3. Methods

3.1. Growth and harvest of piliated bacteria (*see* Note 1)

1. To isolate single colonies of *P. aeruginosa*, streak bacteria from frozen stock stored at −80 °C onto a TSA plate. Incubate plate for 24 h at 30 °C.
2. Identify single colonies with round morphology and smooth margins using a stereo microscope (Figure 1, and *see* Note 8). Streak four colonies onto fresh TSA plates. Incubate for 24 h at 30°C.
3. Select a patch of cells with a sterile swab and resuspend in TSB to reach OD₆₀₀ = 8.0. If at this stage there is already sufficient volume for spreading 50 µL of this bacterial suspension onto 50 plates of TSA (that is, 2.5 mL), skip steps 4 to 6.
4. Grow a robust lawn of bacterial cells by inoculating 100 µL of a cell suspension of OD₆₀₀ = 8.0 onto 5 TSA plates. Grow bacteria for 24 h at 30 °C.

⁷To prepare Tricine-SDS PAGE gel follow method of Schagger (16). We favor this recipe for separating low molecular weight proteins, however any standard SDS PAGE protocol will work.

⁸This is true for the isolation of *Pseudomonas aeruginosa* piliated but non-twitching mutant strains. Colony morphology for piliated strains varies among bacterial strains and species (21, 22).

5. Using a glass spreader and a turntable, remove bacterial lawns from all 5 TSA plates.
6. Transfer bacteria into a 50-mL conical tube with 5 mL of TSB. Use a sterile transfer pipet to scrape cells from the glass spreader into the tube as well as to gently but thoroughly resuspend bacteria. Adjust TSB volume so that the bacteria suspension has an $OD_{600} = 8.0$.
7. Inoculate 50 μ L of this cell suspension onto each of 50 TSA plates and spread using a glass spreader and a turntable. Incubate plates for 24 h at 30 °C.
8. Collect bacterial lawns as above using glass spreader and turntable. Ideally these lawns should be confluent and sticky; they will come off the plate in a goopy clump. We find it convenient to use the same glass spreader for approximately four plates before transferring bacteria into a 250 mL beaker with 25 mL of ice-cold PDB. Use transfer pipet to remove bacteria from glass spreader and to resuspend cells in PDB. Keep adding PDB to suspended bacteria from all 50 plates. A total of 75 mL of PDB should be sufficient. This will be suspension 0 (S0) (Figure 2). During the following procedures, keep bacterial suspension and pili suspension on ice at all times.
9. To dissociate large bacterial clumps, use a transfer pipet and then a 10-mL serological pipet to gently pull cells up and down to achieve a uniform suspension without large clumps. Be patient as bacterial resuspension will increase pili yield, however overly vigorous mixing will lyse cells and contaminate the final pilus preparation with other proteins.
10. After dissociating large bacterial clumps, add a magnetic stir bar and cover the beaker with parafilm. Then, stir the bacterial suspension S0 at medium-low speed in cold-room for one hour.

3.2. Collection of pili

1. Transfer samples to centrifuge tubes with O-ring screw caps. Only fill half of the centrifugation tube to allow good vortexing action.
2. Shear pili from cells by vortexing the cell suspension 3 times in one-minute bursts at maximum strength. Cool on ice for two-minutes between vortexing steps. Collect sample for SDS-PAGE analysis. In choosing a volume to load into gel keep in mind this solution will have a high protein concentration.
3. Sheared pili are now in suspension. To remove cells and cell debris, combine samples to fill and balance centrifuge tubes, and centrifuge samples at $15,000 \times g$ for 20 min (*see* Note 9).
4. Carefully remove supernatant using a serological pipet and transfer to clean centrifuge tubes with O-ring screw caps. Do not disrupt pellet (P1).

⁹The centrifugal force used for these cell-pelleting steps should be high enough to pellet cells but not high enough to lyse cells or pellet disaggregated pilus filaments. $15,000 \times g$ is a good guide however lower rcf ($8-10,000 \times g$) for a longer time (30 min) may be sufficient. It is worthwhile to follow pilin protein by SDS page to ensure pili are not spinning down with cells.

5. Centrifuge the supernatant a second time to remove residual cells at $15,000 \times g$ for 10 min. (*see* Note 9). Transfer supernatant (S1) containing sheared pili to a clean disposable tube. Set aside a sample of the supernatant for analysis.
6. Prepare a 16% Tricine-SDS PAGE gel following reference (16).
7. Run samples to confirm successful separation of pili from cell fractions.

3.3. Purification of pili

1. Prepare dialysis membrane according to manufacture's instructions.
2. Load sheared pili sample S1 into dialysis tubing and dialyze against 4 L of cold PBB for 4 h at 4°C. Stir at low speed to facilitate buffer exchange.
3. Change dialysis buffer and repeat dialysis procedure until the pH of the sample reaches pH 7.5 (*see* Note 10).
4. Upon neutralization of pH during dialysis, the pilus filaments will aggregate by bundling. Carefully remove sample from dialysis tubing and transfer it into a centrifuge tube. For maximum yield, rinse the inside of the tubing with PBB and add to sample. Keep an aliquot for analysis.
5. To collect aggregated pili, centrifuge sample at $20,000 \times g$ for 40 min.
6. Remove supernatant (S2) using serological pipet. Invert tubes and drain onto paper towel. At this step, the pellet (P2) contains sheared pili, and its appearance should be white. (If this pellet contains pink, it includes some residual cells.) Resuspend the pelleted pili (P2) in PDB, starting with ~3 mL. Add PDB as needed to make a non-viscous solution.
7. Optionally, centrifuge to remove contaminants not yet in suspension, which will pellet (P3) while pili remain in supernatant (S3).
8. Repeat dialysis procedure, now dialyzing S3 against PBB.
9. Collect purified pili by centrifugation; remove supernatant S4, and re-suspend pellet (P4) in desired final buffer to reach appropriate concentration (for example, 5 mL) (*see* Notes 11 and 12).

3.4. Assessment of results

1. To monitor pilus purity, run pilus preparation samples in 16% Tricine-SDS PAGE gel (Figure 2a, Table 1) and visualize by Coomassie blue or silver staining. The latter also permits estimation of LPS contamination. Yields of ~0.2

¹⁰For best success, actually check the pH of the solution inside the dialysis bag. The lowering of salt together with neutralization of pH is the fundamental basis of the method and we have found that failure to reach equilibrium at this stage leads to very low pilus yield.

¹¹To achieve the desired purity, repeat re-suspension and aggregation cycles. One modification to this procedure is at the last step to bring the cold solution to a final concentration of 20% ammonium sulfate and stir it for 2 h to precipitate pili while leaving contaminating flagellar filaments in solution (20).

¹²Depending on the application one can remove residual LPS using polymixin B agarose beads (our unpublished result).

to 0.5 mg of pili per plate at the S3 step can be achieved; although the overall yield of pilin falls with each cycle the purity increases (Figure 3A, Table 1).

2. Negative staining can be used for visualization of pilus filaments using transmission electron microscopy (Figure 2B, C).

Acknowledgments

We are grateful to Dr. Nicole Koropatkin for purifying grams of pili and optimizing this protocol in the process, and to Dr. Lisa Craig for many years of collegial interactions, and helpful suggestions for this chapter.

References

1. Hansen JK, Forest KT. Type IV pilin structures: insights on shared architecture, fiber assembly, receptor binding and type II secretion. *J Mol Microbiol Biotechnol*. 2006; 11:192–207. [PubMed: 16983195]
2. MacRae TH, Dobson WJ, McCurdy HD. Fimbriation in gliding bacteria. *Can J Microbiol*. 1977; 23:1096–1108. [PubMed: 407994]
3. Henrichsen J, Blom J. Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility. *Acta Pathol Microbiol Scand B*. 1975; 83:161–170. [PubMed: 1155114]
4. Swanson J. Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J Exp Med*. 1973; 137:571–589. [PubMed: 4631989]
5. Saarimaa C, Peltola M, Raulio M, Neu TR, Salkinoja-Salonen MS, Neubauer P. Characterization of adhesion threads of *Deinococcus geothermalis* as type IV pili. *J Bacteriol*. 2006; 188:7016–7021. [PubMed: 16980504]
6. Imam S, Chen Z, Roos DS, Pohlschroder M. Identification of surprisingly diverse type IV pili, across a broad range of gram-positive bacteria. *PLoS One*. 2011; 6:e28919. [PubMed: 22216142]
7. Melville S, Craig L. Type IV pili in Gram-positive bacteria. *Microbiol Mol Biol Rev*. 2013; 77:323–41. [PubMed: 24006467]
8. Berry JL, Pelicic V. Exceptionally widespread nanomachines composed of type IV pilins: the prokaryotic Swiss Army knives. *FEMS Microbiol Rev*. 2015; 39:134–154. [PubMed: 25793961]
9. Pelicic V. Type IV pili: e pluribus unum? *Mol Microbiol*. 2008; 68:827–837. [PubMed: 18399938]
10. Brinton, CC., Bryan, J., Dillon, J.-A., Guerina, N., Jen Jacobson, L., Labik, A., Lee, S., McMichael, J., Polen, S., Rogers, K., To, ACC., To, SCM. Uses of pili in Gonorrhea control: role of bacterial pili in disease, purification and properties of Gonococcal pilus vaccine for Gonorrhea. In: Brooks, GF., editor. *Immunobiology of Neisseria gonorrhoeae: proceedings of a conference held in San Francisco, California, American Society for Microbiology; Washington D.C.* 1978.
11. Li J, Lim MS, Li S, Brock M, Pique ME, Woods VL Jr, Craig L. *Vibrio cholerae* toxin-coregulated pilus structure analyzed by hydrogen/deuterium exchange mass spectrometry. *Structure*. 2008; 16:137–148. [PubMed: 18184591]
12. Forest KT, Dunham SA, Koomey M, Tainer JA. Crystallographic structure reveals phosphorylated pilin from *Neisseria*: phosphoserine sites modify type IV pilus surface chemistry and fibre morphology. *Mol Microbiol*. 1999; 31:743–752. [PubMed: 10048019]
13. Parge HE, Bernstein SL, Deal CD, McRee DE, Christensen D, Capozza MA, Kays BW, Fieser TM, Draper D, So M. Biochemical purification and crystallographic characterization of the fiber-forming protein pilin from *Neisseria gonorrhoeae*. *Journal of Biological Chemistry*. 1990; 265:2278–2285. [PubMed: 1967608]
14. Parge HE, Forest KT, Hickey MJ, Christensen DA, Getzoff ED, Tainer JA. Structure of the fibre-forming protein pilin at 2.6 Å resolution. *Nature*. 1995; 378:32–38. [PubMed: 7477282]
15. Craig L, Taylor RK, Pique ME, Adair BD, Arvai AS, Singh M, Lloyd SJ, Shin DS, Getzoff ED, Yeager M, Forest KT, Tainer JA. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. *Mol Cell*. 2003; 11:1139–1150. [PubMed: 12769840]

16. Schagger H. Tricine-SDS-PAGE. *Nat Protoc.* 2006; 1:16–22. [PubMed: 17406207]
17. Bradley DE. The adsorption of *Pseudomonas aeruginosa* pilus-dependent bacteriophages to a host mutant with nonretractile pili. *Virology.* 1974; 58:149–163. [PubMed: 4206974]
18. Whitchurch CB, Hobbs M, Livingston SP, Krishnapillai V, Mattick JS. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene.* 1991; 101:33–44. [PubMed: 1676385]
19. Bradley DE. A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. *Can J Microbiol.* 1980; 26:146–154. [PubMed: 6105908]
20. Frost LS, Paranchych W. Composition and molecular weight of pili purified from *Pseudomonas aeruginosa* K. *J Bacteriol.* 1977; 131:259–269. [PubMed: 406258]
21. Han X, Kennan RM, Davies JK, Reddacliff LA, Dhungyel OP, Whittington RJ, Turnbull L, Whitchurch CB, Rood JJ. Twitching motility is essential for virulence in *Dichelobacter nodosus*. *J Bacteriol.* 2008; 190:3323–3335. [PubMed: 18310333]
22. Meng Y, Li Y, Galvani CD, Hao G, Turner JN, Burr TJ, Hoch HC. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *J Bacteriol.* 2005; 187:5560–5567. [PubMed: 16077100]
23. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods.* 2012; 9:671–5. [PubMed: 22930834]
24. Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, Duvaud S, Flegel V, Fortier A, Gasteiger E, Grosdidier A, Hernandez C, Ioannidis V, Kuznetsov D, Liechti R, Moretti S, Mostaguir K, Redaschi N, Rossier G, Xenarios I, Stockinger H. ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Res.* 2012; 40:W597–603. [PubMed: 22661580]

Figure 1. Colony morphologies. *P. aeruginosa* strain K *pilT* is plated on 1.5% agar TSB plates. Piliated colonies have a smooth, domed appearance (black arrow). Avoid flat, spreading colonies (white arrow).

Figure 2.

Workflow for T4aP isolation. Bacteria are suspended in PDB and pili are sheared by vortexing (S0). Cell debris and bacteria are removed by centrifugation (P1). The remaining solubilized pili (S1) are aggregated by dialyzing against PBB, and collected by centrifugation (P2). For purification purposes, pili in P2 are resuspended in PDB (S3) and aggregated by another dialysis against PBB (P4). Cycles of re-suspension and aggregation can be repeated to improve pilus purity although at a loss of overall yield.

Figure 3. Pili isolated from *P. aeruginosa* strain K *pilT*. (A) Proteins were separated in 16% Tricine-SDS PAGE gel (molecular mass markers are in kDa). (B) Whole cells and (C) purified pili (plus contaminating flagella) analyzed by negative staining and transmission electron microscopy. (Scale bars: 100 nm)

Table 1

Purification yields at each step. Approximate purity was estimated by image analysis of the Coomassie-stained gel in Figure 3a using ImageJ [23]. Protein concentration was estimated using the OD₂₈₀ and a calculated extinction coefficient for pilin of 14,100 M⁻¹ cm⁻¹ (as calculated using ExPasy ProtParam [24]). We present data for two representative examples of yield from the protocol described here. The left hand columns are based on a purification by a relatively inexperienced researcher using 44 plates with volumes exactly as described in protocol (and shown in Figure 3a). The right hand columns are based on a purification by a more practiced investigator from 32 plates with correspondingly reduced volumes.

	P3		S3		P4		S4	
Total protein	(1 mg)	(0.9 mg)	16 mg/l	30.5 mg	10 mg	11.7 mg	(6.4 mg)	(12.6 mg)
Purity	84%	92%	71%	88%	81%	98%	70%	82%
Pilin yield (loss)	(1 mg)	(0.5 mg)	11 mg	26.8 mg	8 mg	11.5 mg	(4.5 mg)	(14.5 mg)

¹Note in this case the S3 concentration was estimated from the dialysate after S3 dialysis against PBBB rather than in the initial S3. Thus this value is an underestimate of the true yield at the S3 stage.