Supplemental Figures

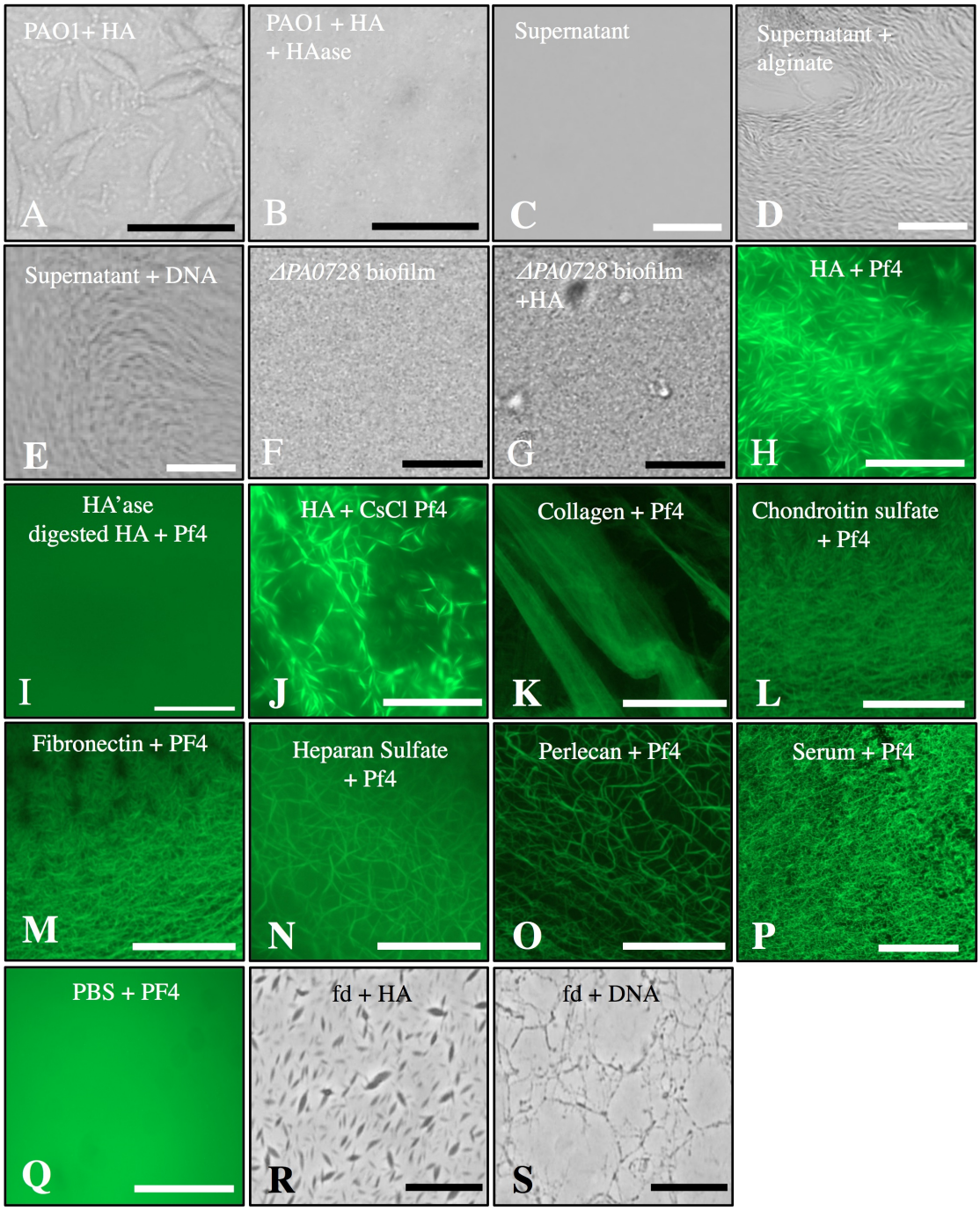

Figure S1. Interactions Between Diverse Polymers and Filamentous Pf Phage Result in the Assembly of Large Interwoven Structures, Related to Figure 1.

(A-B) Supplementation of PAO1 biofilm cultures with exogenous HA (5 mg/ml) results in the assembly of distinct structures. Treatment of these structures with hyaluronidase (HA'ase) results in the destruction of the structures. Scale bars, 50 μm.

(C-E) Biofilm supernatants form interwoven sheets when mixed with alginate or DNA. Scale bars, 50 μm.

(F-G) Supplementation of *ΔPA0728* biofilm cultures with exogenous HA does not lead to structure formation. Scale bars, 50 μm.

(H-I) Fluorescently labeled Pf4 (PEG purified, green, 8.8 x 10^9 PFU/ml) and HA (5 mg/ml) interact to assemble structures. Treatment of these structures with hyaluronidase (HA'ase) results in the destruction of the structures.

(J) Fluorescently labeled Pf4 (CsCl purified, green, 1.0 x 10^{10} PFU/ml) mixed with 5 mg/ml form similar structures to PEG purified Pf4. Scale bars, 50 μm.

(K-Q) Fluorescently labeled Pf4 (green, 8.8 x 10^9 PFU/ml) mixed with 5 mg/ml of the indicated polymer (or PBS in (Q)) were imaged by fluorescent microscopy. Polymers in the absence of Pf4 did not display any structures, not shown. Scale bars, 50 μm except (P) where the scale bar is 200 μm.
(R and S) The filamentous phage fd, which is produced by *E. coli*, forms similar structures when mixed with HA or DNA. Scale bars, 20 μm.

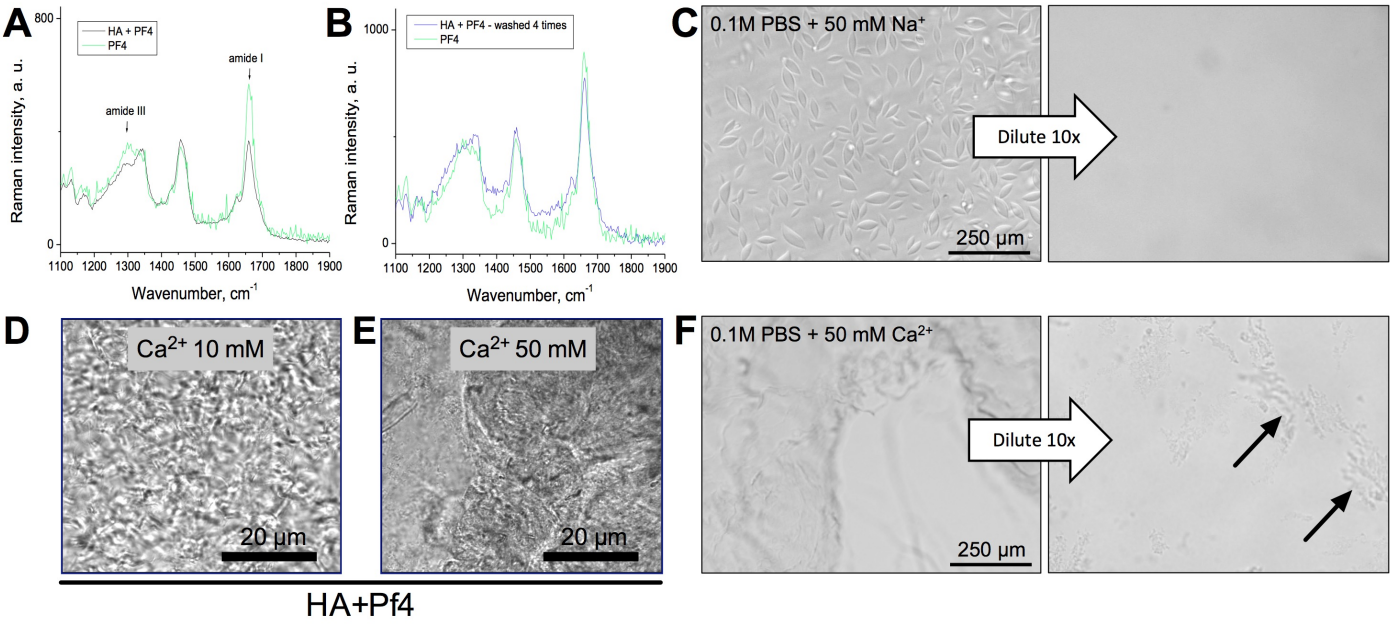

Figure S2. The Formation and Dissolution of Structures in Mixtures of Pf4 and HA are Dependent on the Valency and Concentration of Ions, Related to Figure 1.
(A) Raman spectra of amide band regions indicate tight interactions of Pf4 and HA, resulting in suppressed β-sheet peaks of Pf4.
(B) The suppression of β-sheet peaks is weakened after removing salts by washing of the sample.
(C) Structures formed in the setting of 50mM of the monovalent cation Na^+ disappear upon 10x dilution in DI water.
(D and E) Large aggregates form when the divalent cation calcium (Ca^{2+}) is added to mixtures of HA and Pf4.
(F) Structures formed in the setting of 50mM of the divalent cation Ca^{2+} persist upon 10x dilution in DI water.

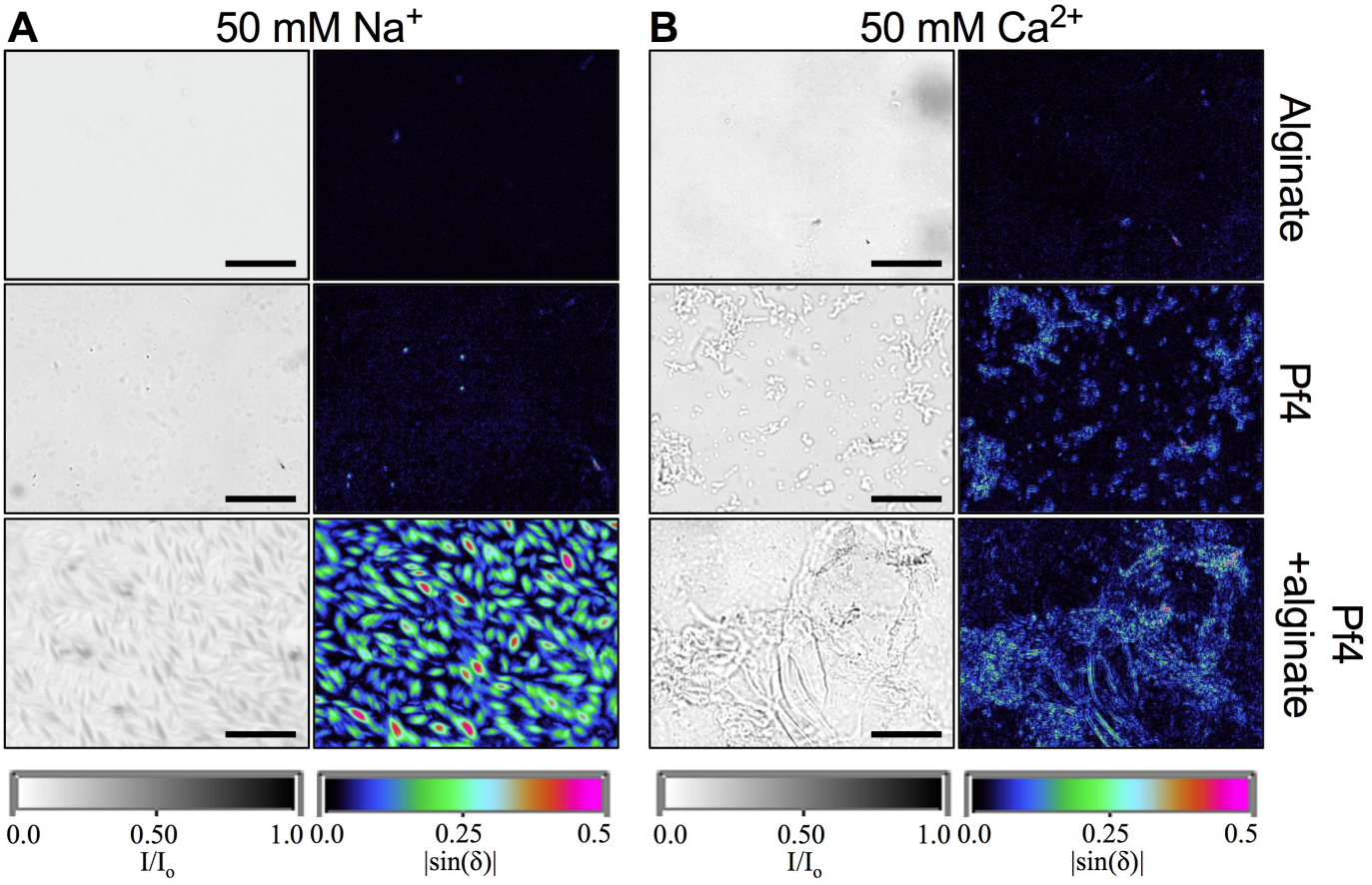

Figure S3. Mixtures of Alginate and the Monovalent Cation Na^+ Form Birefringent Liquid Crystals While Alginate and the Divalent Cation Ca^{2+} Form Large Aggregates That Are Minimally Birefringent, Related to Figure 2.

The birefringence of alginate (10 mg/ml) and Pf4 (1 x 10^{10} PFU/ml) mixed 1:1 (vol:vol) was imaged ($|\sin(\delta)|$) in the presence of 50 mM NaCl or $CaCl_2$. Transmitted light is displayed as I/I_o where I = the intensity of light emerging from the sample and I_o = the intensity of incident light. Scale bars, 10 μm.

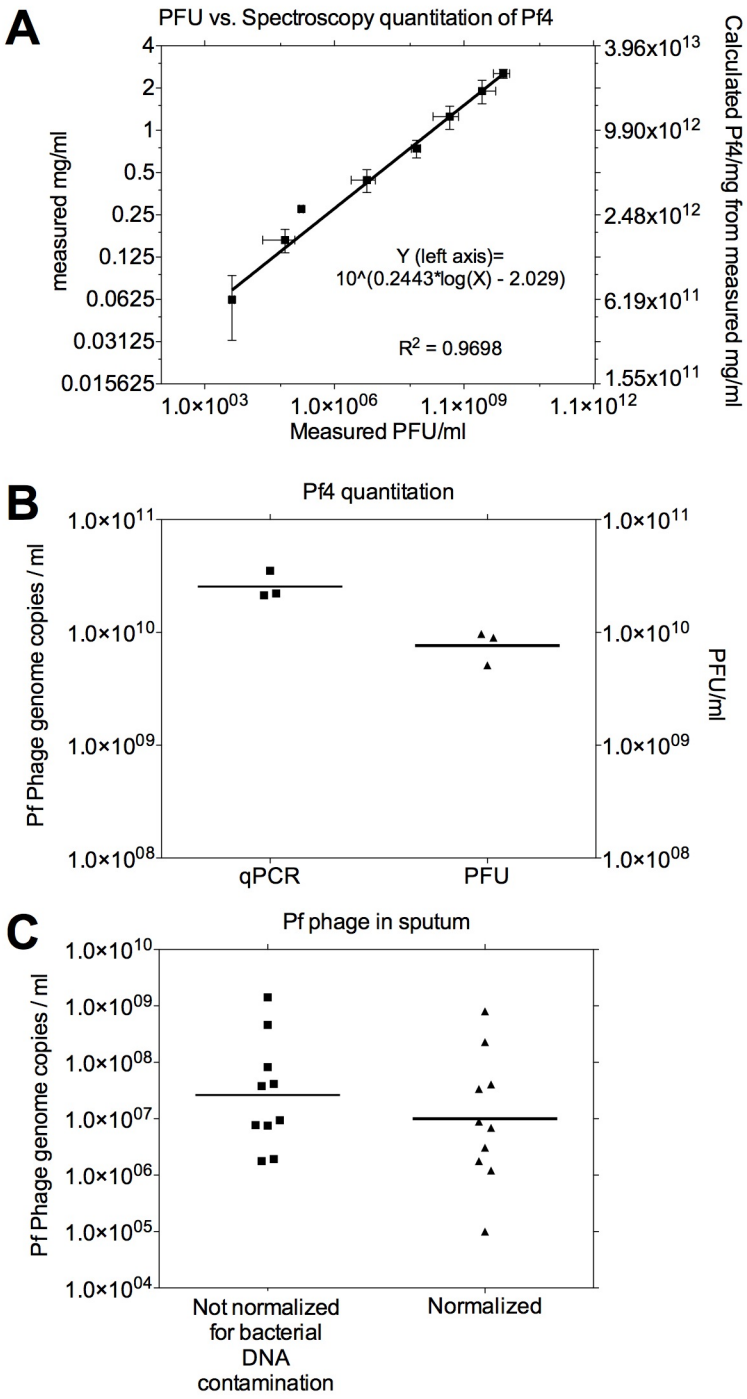

Figure S4. Quantitation of Pf Phage by PFU, spectroscopy, and qPCR, Related to Figure 3.

(A) A 2x dilution series of Pf4 was quantified by PFU enumeration (X-axis) and by spectroscopy (Left Y-axis). Pf numbers (Right Y-axis) were calculated from the measured spectroscopy values using a molecular weight for Pf4 of 60.8 MDa, which corresponds to 9.9 x 10^{12} Pf4 particles/mg. Results are mean ± SD of 3 experiments.
(B) Quantitation of Pf4 by qPCR and PFU was compared. Results are plotted as the geometric mean of three experiments.
(C) Pf phage quantified by qPCR in sputum collected from CF patients infected by *P. aeruginosa* (n=10) are shown pre and post-normalization for contaminating bacterial chromosomal DNA, see Supplemental Experimental Procedures for details. Results are plotted as the geometric mean.

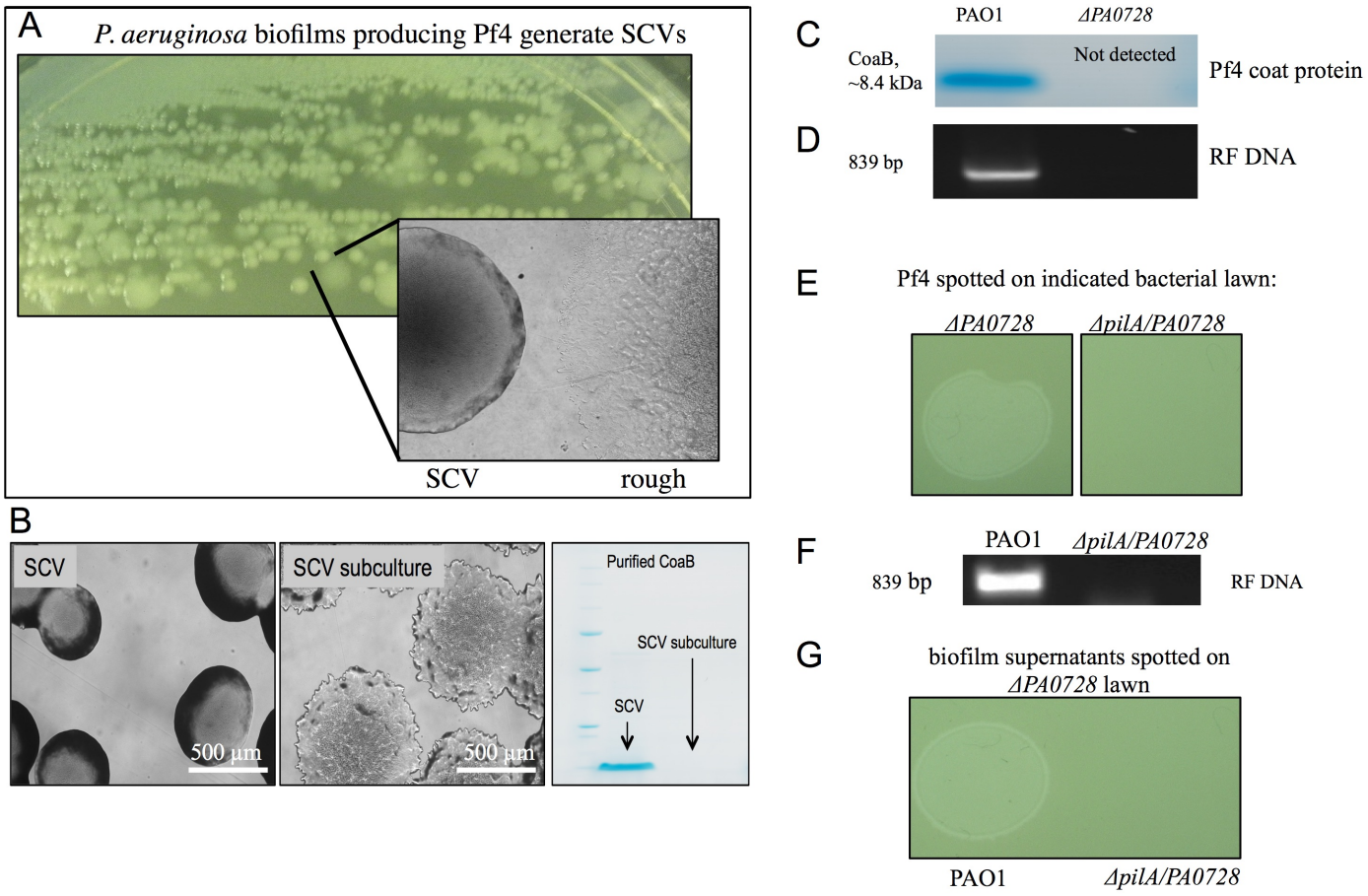

Figure S5. Small Colony Variant Production, Phage Production, and Phage Sensitivity of Various Strains of *P. aeruginosa,* Related to Figures 5 and 6.

(A) *P. aeruginosa* PAO1 biofilms producing Pf4 generate both wild type "rough" colonies with rough edges and small colony variants (SCVs). Isolates were collected by dipping an inoculating loop into the biofilm followed by isolating for individual colonies by streaking on an LB agar plate.

(B) When the SCV strain is subcultured, it reverts back to a rough morphology (left and middle panels). This coincides with reduced Pf4 production, as shown by SDS-PAGE analysis for CoaB purified from culture supernatants (right panel).

(C) Δ*PA0728* biofilm supernatants do not contain the major coat protein of Pf4, CoaB, as analyzed by SDS-PAGE and confirmed by mass spectrometry.

(D) Pf4 DNA packaged within mature phage particles were detected by PCR primers that can only amplify circularized Pf4 DNA (the replicative form, RF).

(E) Purified Pf4 (8.8 x 10^9 PFU/ml) was spotted onto lawns of the indicated strains of *P. aeruginosa*.

(F) Circular Pf4 DNA was not detected in Δ*PA0728/pilA* biofilm supernatants by PCR using the RF specific primers.

(G) Supernatants collected from Δ*PA0728/pilA* biofilms did not produce any plaques on a lawn of Δ*PA0728*.

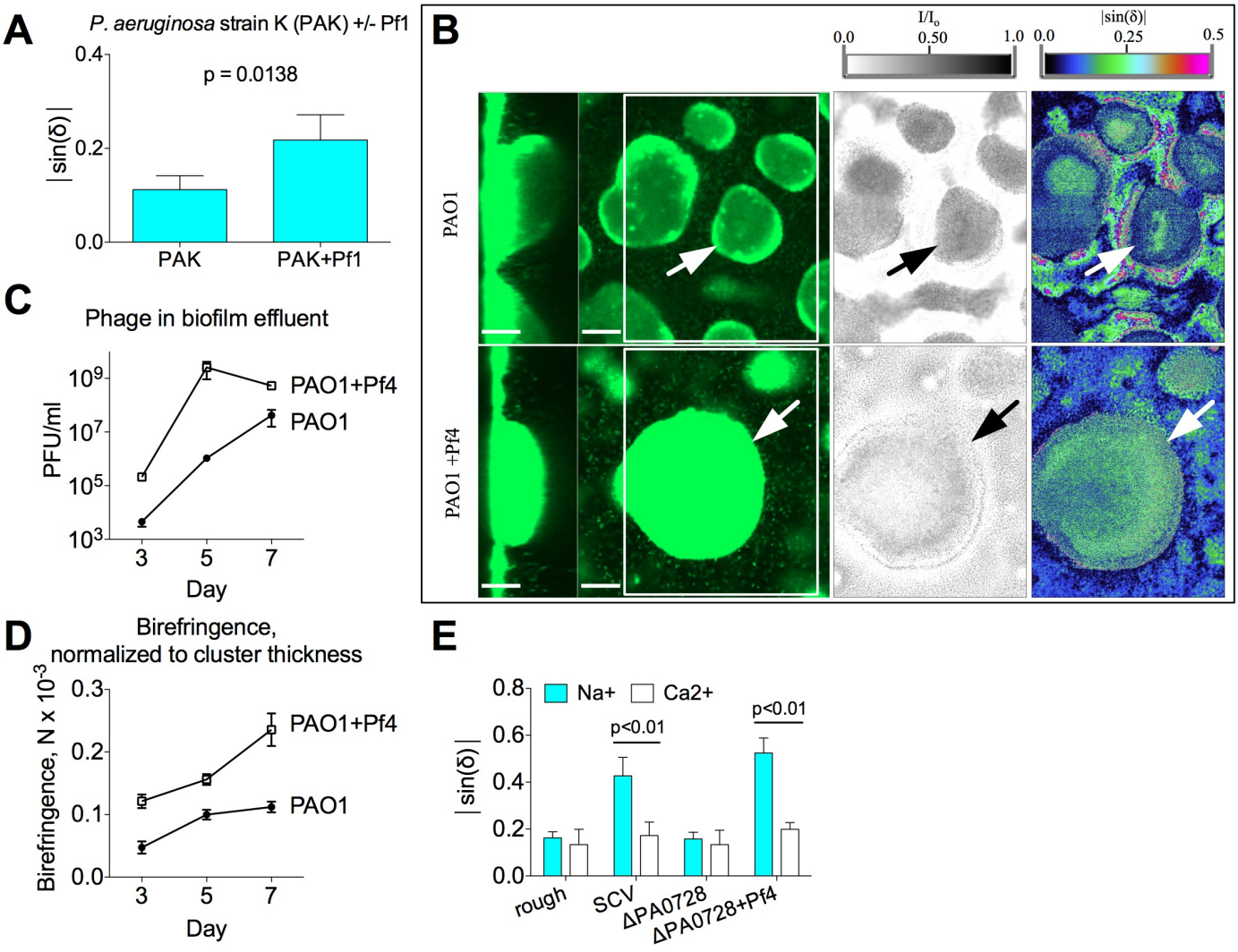

Figure S6. Birefringence analysis of *P. aeruginosa* flowcell biofilms, Related to Figure 4.
(A) Birefringence (|sin(δ)|) of PAK colony biofilms +/- Pf4 was quantified after normalizing for sample thickness. Results are mean ± SD of 3 experiments.
(B) The Rotopol imaging system was coupled with confocal microscopy to image PAO1 and PAO1 + Pf4 flowcell biofilms after three days of growth. Biofilms were stained for biomass using Syto9 (green) and imaged using confocal microscopy. Birefringence was then measured in the same field of view using Rotopol. Arrows indicate representative clusters for which birefringence was measured in (D). Scale bar = 35 μm.
(C) Phage production was quantified in the biofilm effluent over time by plating for PFUs on a lawn of Δ*PA0728*. Results are mean ± SD of 3 experiments.
(D) Birefringence was measured over time and normalized to cluster height. Cluster heights were measured in the Z-axis by confocal microscopy, which were then used to normalize birefringence measurements to cluster height using the formula $\Delta n=\delta\lambda/2\pi L$ where Δn=birefringence, L optical path (cluster height measured by confocal microscopy), and λ=wavelength (550 nm). Results are mean ± SD of 3 experiments.
(E) Birefringence (|sin(δ)|) of colony biofilms grown on LB agar plates (+/- 10 mM Na^+ or 10 mM Ca^{2+}) was quantified after normalizing for sample thickness. Results are mean ± SD of 4 experiments.

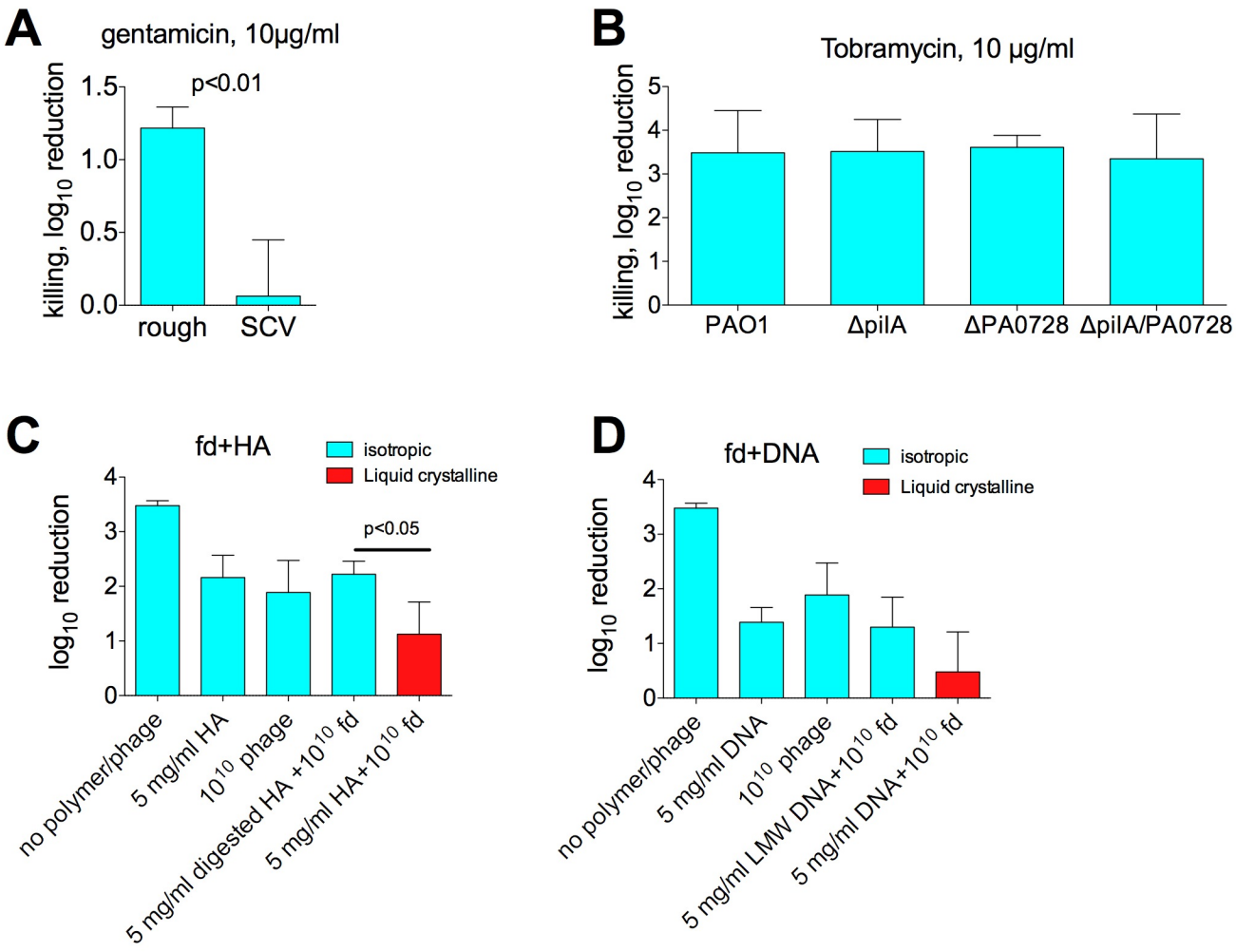

Figure S7. The Filamentous Phage Fd Offers *P. aeruginosa* Protection From Tobramycin by Interacting With Polymers to Form Liquid Crystals, Related to Figure 6 and 7.
(A) Log_{10} reduction in killing of SCV versus rough PAO1 colony biofilms by 10 μg/ml gentamicin. Results are mean ± SD of 3 experiments.
(B) Log_{10} reduction in killing of Δ*PA0728*, Δ*pilA*, and Δ*PA0728*/*pilA*, and control PAO1 strains by 10 μg/ml tobramycin. Results are mean ± SD of 3 experiments.
(C and D) Isotropic mixtures of (C) fd and HA or (D) fd and DNA offer *P. aeruginosa* Δ*PA0728* some protection against 10 μg/ml tobramycin (90 minute treatment time). Concentrations of fd and polymer where liquid crystals form offer the most protection (red bars). Replacing the polymer in the liquid crystal mixtures with low molecular weight oligomers (resulting in an isotropic rather than liquid crystalline mixture) results in a loss of the extra protection offered by liquid crystal assembly. Results are mean ± SD of 3 experiments.

Movie S1. Visualization of Pf4 Mediated Liquid Crystal Assembly, Related to Figure 1.
Formation of liquid crystals immediately after mixing equal volumes of 5.5 x 10^{11} PFU/ml Pf4 and 10 mg/ml HA, as observed by light microscopy. Duration, 84 seconds.

Movie S2. Visualization through crossed polarizing lenses of *P. aeruginosa* SCV colonies placed between a glass slide and coverslip to normalize for thickness. Related to Figure 4.

Movie S3. Visualization through crossed polarizing lenses of *P. aeruginosa* "rough" wild type colonies placed between a glass slide and coverslip to normalize for thickness. Related to Figure 4.

Table S1. Bacterial Strains, Plasmids, and PCR Primers Used in this Study, Related to Experimental Procedures.

Strain or plasmid	Genotype or description	Source
P. aeruginosa		
PAO1	Wild type	(Holloway, 1955)
SCV	Isolated from a PAO1 biofilm. The SCV is deficient in twitching motility but swim-motile.	This study
PAK	Wild type	ATCC 25102
ΔPA0728	PAO1 Δ*PA0728*	This study
ΔpilA	PAO1 Δ*pilA*	(Zhao et al., 2013)
ΔPA0728/pilA	PAO1 Δ*PA0728/pilA*	This study
E. coli		
DH5α		Invitrogen
ATCC 15669	Male specific bacteriophage (fd) host	ATCC 15669
Bacteriophage		
Pf4	Isolated from PAO1 biofilms	This study
Pf1	Filamentous bacteriophage that infects PAK	ATCC 25102-B1
Fd	Filamentous bacteriophage that infects *E. coli*	ATCC 15669-B2
Plasmids		
pEX-Δ*PA0728*	Deletion construct targeting PA0728	(Castang and Dove, 2012)
qPCR standard Pf conserve	Sequence amplified by Pf-Conserve primers placed into pCR2.1 backbone	This study
qPCR standard 16S	Sequence amplified by rpIU primers placed into pCR2.1 backbone	This study
Primers		
Pf-ConserveF	GTC CCC GTT CCT TCT GCG	This study
Pf-ConserveR	CGC TGT CGT ACA CAA CGT G	This study
rplU-F	CGT CAC GAC AAG GTC CGC	This study
rplU-R	GGC CTG AAT GCC GGT GAT C	This study
Pf4F	ATT GCT TCA TCG CGC TGC T	(Webb et al., 2004)
Pf4R	TCC AGT CAC AAA TGG CCT CTA	(Webb et al., 2004)
PA0728F	CGG TCT ACG ATC CGT TCT GG	This study
PA0728R	CTC CAC CAC GCT CCA TAT CC	This study

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Biofilm experiments

Static biofilms were grown in LB broth (supplemented with polymer where indicated) at 37°C in 6-well culture plates. Static biofilms were inoculated with 50 μl of an overnight culture. The media (2 ml per well) was exchanged every 24 h for up to 14 days. Rough and SCVs were isolated from static biofilms by dipping an inoculating loop into the biofilm and streaking an LB plate. Supernatants were collected from static biofilms and bacterial cells were removed by centrifuging at 9,000g for 5 minutes. Supernatants were then passed through a 0.2 syringe filter. Proteins in the supernatants were analyzed by mass spectrometry as described previously (Hood et al., 2010). Viable biofilm bacteria were resuspended into 1 ml PBS by thorough vortexing, homogenized by passing the sample through a 20-gauge needle multiple times, serially diluted, and plated onto LB agar to enumerate viable CFUs.

Construction of strains *ΔPA0728* and *ΔPA0728/pilA*

Plasmid pEX-*ΔPA0728* (Castang and Dove, 2012) was introduced to PAO1 or *ΔpilA* (Table S1) to create strains *ΔPA0728* and *ΔPA0728/pilA*, respectively, by allelic exchange (Hoang et al., 1998). Deletions were confirmed by sequencing using primers PA0728-seq-F and PA0728-seq-R and a primer set specific to an internal region of the *PA0728* gene (PA0728-F and PA0728-R) to rule out meroploidy (Table S1).

Phage purification

Bacteriophage were purified by precipitation with polyethylene glycol (PEG) as previously described (Boulanger, 2009). Briefly, bacterial supernatants containing phage were treated with 10 μg/ml DNase (Sigma-Aldrich Co., St. Louis, MO) for 2 h at 37°C. Next, phage were precipitated from the supernatant by adding 0.5 M NaCl followed by 10% (w/vol) PEG 6000 (Sigma-Aldrich Co., St. Louis, MO) followed by an overnight incubation at 4°C. Phage were pelleted by centrifugation at 12,000 g for 20 minutes at 4°C. The pellet was re-suspended in PBS and subjected to two additional rounds of PEG precipitation. Finally, phage pellets were re-suspended in PBS and thoroughly dialyzed against PBS using a 10 kDa molecular weight cutoff Slide-A-Lyzer dialysis cassettes (Thermo Scientific, Waltham, Massachusetts).

Adhesion

Adhesion of *P. aeruginosa* to polystyrene microtiter plates was performed as described (Webb et al., 2004) with modifications. Overnight cultures were diluted to an OD_{600} of 0.1 in LB broth and 150 μl was placed into 96-well microtiter plates. PAO1 or *ΔPA0728* were supplemented with ~10^6 PFU/ml Pf4, resulting in active phage production and a final Pf4 titer of ~10^9 PFU/ml. PAK was supplemented with 8.8 x 10^9 PFU/ml Pf4 and *ΔPA0728/pilA* was supplemented with the indicated concentrations of purified fd phage. After 24 h at 37°C, non-adherent bacteria were removed by inverting the plate. Cells were washed once by gently submerging the plate into deionized water. After this, 175 μl of a 1% solution of crystal violet (CV) was added to each well followed by a 15-minute incubation period at room temperature. Wells were washed twice with water and the remaining CV was solubilized by adding 200 μl of 20% acetic acid to each well. Following a 15-minute incubation at room temperature, 100 μl was transferred to a fresh plate and the absorbance (550 nm) was measured using a plate reader.

Viscosity measurements

Viscosity was measured with a capillary viscometer (Cannon Instrument Company, State College, PA) at room temperature following the manufacturers instructions as described in the Supplemental Experimental Procedures.

AFM imaging

AFM imaging was performed with a NT-MDT NTEGRA Spectra ® AFM-Raman integrated instrument or NX-10 AFM from Park Systems in non-contact mode using standard commercial cantilevers from Miscromasch® with less than 30% oscillation damping. Polished Si wafers from VWR were used as sample substrates. Data analysis of AFM imaging was done in NT-MDT™ integrated Nova® data analysis software (v. 1.1.0.1820), XEI™ (v. 1.8.1.Build214) and Gwyddion (v. 2.31).

Raman spectroscopy

The Raman measurements were performed using backscattering geometry on phage and phage-polymer mixed samples, dried in air on titanium foil. The illumination source was a 473 nm Cobolt Blues™ continuous wave diode-pumped solid-state laser brought to the sample by a Mitutoyo long working distance objective (100X, 0.7 NA). Laser power at sample was 1.08 mW, as measured by Coherent LaserCheck™ . The spectra were recorded for 600 s or sometimes longer, until sufficient counts were accumulated. Raman spectra were acquired in 10 min sequential acquisitions with an Andor CCD peltier-cooled camera (as part of a NTEGRA Spectra system) with a 600 grating from solid samples. Each spectrum was monitored for signs of sample degradation and discarded if any were observed. Optical images of the sample were obtained through the same objective. Raman spectra were baseline corrected to account for fluorescent background.

Phase diagrams

Mixtures of Pf4 and indicated polymers were prepared at the indicated concentrations. After a 60-minute incubation, the presence or absence of liquid crystals was visually scored using a microscope.

Phage quantitation by qPCR

Ten μl reaction volumes contained 5 μl SYBR Select Master Mix (Life Technologies, Grand Island, NY), 100 nM of each primer, and 2 μl template. Cycling conditions were as follows: 50°C 2min, 95°C 2min, (95°C, 15 sec, 60°C 1 minute) x 40 cycles. Template copy numbers were calculated by constructing a standard curve from plasmids containing the amplified sequence (Table S1).

To control for any contamination by *P. aeruginosa* chromosomal DNA that might contain Pf prophage sequences, 50S copy numbers were subtracted from Pf copy numbers. Normalized and un-normalized quantitation of Pf phage in sputum by qPCR is presented in Figure S4C.

Antibiotic binding experiments

MICs were determined by adding increasing amounts of tobramycin or ciprofloxacin to the indicated concentrations of Pf4 and DNA (prepared in LB broth) in 96-well plates (100 μl volumes). Samples were incubated at room temperature for 4-h to allow binding. An overnight culture of *E. coli* DH5α was diluted to an OD_{600} of 0.05 in LB broth and 10 μl was added to each well. The cultures were sealed with a breathable membrane and incubated overnight at 37°C. The following day, the highest concentration at which growth occurred was plotted.

Sequestration experiments were performed by placing 200 μg/ml tobramycin with the indicated mixtures of Pf4 and DNA in a 2 kDa (0.5 ml volume) slide-A-Lyzer dialysis cassette (Thermo). Cassettes were dialyzed against PBS. PBS was collected and analyzed for the presence of unbound tobramycin by HPLC-MS, as described below.

Cy5 conjugated tobramycin (a gift from B.S. Tseng) was added to the indicated phage and DNA mixtures (Pf4, 10^{10} PFU/ml, DNA, 2.5 mg/ml) and incubated at room temperature for four hours. The samples were then imaged by fluorescent microscopy.

Detection of tobramycin by HPLC-MS

HPLC-MS/MS analysis. Preparation of HPLC-MS/MS standards. All chemicals and solvents for HPLC-MS/MS were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Hampton, NH). Stock standard solutions of tobramycin were prepared by dilution of accurately weighed powders in DI water. Calibration spiking solutions were prepared by diluting the stock solution with DI water to final concentrations of 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/ml of DFO. Chromatograms for standards were used to establish characteristic retention times (RTs) of tobramycin, and verified that the MS signal was linear over the range. The peak areas of tobramycin were calculated and plotted against the concentration of the calibration standards. Calibration curves were generated using the least squares linear regression method with Analyst® 1.5.1 software.

HPLC-MS/MS data acquisition. For DFO separation and detection, the flow rate was set at 300 μl/min. Chromatographic separation was performed on an Ascentics ES Cyno column (Sigma, St. Louis, MO). Mobile phase A was 5 mM ammonium acetate/0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. A 2.5-min elution was performed with a 20-90% gradient of mobile phase B. After 3 min, %B was changed to 20% and kept for 1 min. The HPLC was directly coupled to an AB SCIEX 4000 QTRAP triple quadrupole mass spectrometer with electrospray ionization. To monitor tobramycin, the mass spectrometer was operated in the positive multiple reactions monitoring mode, with transitions of 561.17/102.30 and 560.79/201.00 Da. The switching valve diverted HPLC flow to the mass spectrometer at 0-1 min. The elution time for tobramycin was 0.35 min.

HPLC-MS/MS data analysis. Peak detection, integration and data processing were performed with the AB SCIEX Analyst 1.5.1 software package. Concentrations of tobramycin were calculated by plotting the peak area of unknown samples against the calibration curve prepared in the corresponding matrix. A 1/x weighted linear regression was used to calculate the unknown tobramycin concentrations.

Movies

Movies of Pf4 interacting with hyaluronan were acquired using a Canon EOS Rebel T5i camera mounted on a Zeiss Primo Vert inverted light microscope. On a clean glass microscopy slide, 2 μL Pf4 (PEG-purified, 5 x 10^{11} PFU/ml in PBS) was added to 2 μl HA (Lifecore Biomedical, 1.20MDa-1.80MDa, 10 mg/ml in PBS) and the sample was recorded for 84 seconds at 40x magnification. Imaging started immediately after addition of the HA. The original footage was processed with Canon EOS Utility software and modified using Apple iMovie software to adjust the brightness and increase the speed 5.6x to 15 seconds.

SUPPLEMENTAL REFERENCES

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