Specific Disruption of Established *P. aeruginosa* Biofilms Using Polymer-Attacking Enzymes

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Abstract

Biofilms are communities of bacteria embedded in a polymeric matrix which are found in infections and in environments outside the body. Breaking down the matrix renders biofilms more susceptible to physical disruption and to treatments such as antibiotics. Different species of bacteria, and different strains within the same species, produce different types of matrix polymers – this suggests that targeting specific polymers for disruption may be more effective than non-specific approaches to disrupting biofilm matrices. In this study, we treated *Pseudomonas aeruginosa* biofilms with enzymes that are specific to different matrix polymers. We measured the resulting alteration in biofilm

mechanics using bulk rheology, and changes in structure using electron microscopy. We find that, for biofilms grown *in vitro*, the effect of enzymatic treatment is greatest when the enzyme is specific to a dominant matrix polymer. Specifically-matched enzymatic treatment tends to reduce yield strain and yield stress and increase the rate of biofilm drying, due to increased diffusivity as a result of network compromise. Electron micrographs qualitatively suggest that well-matched enzymatic treatments reduce long-range structure and shorten connecting network fibers. Previous work has shown that generic glycoside hydrolases can cause dispersal of bacteria from *in vivo* and *ex vivo* biofilms into a free-swimming state, and thereby make antibiotic treatment more effective. For biofilms grown in wounded mice, we find that well-matched treatments that result in the greatest mechanical compromise *in vitro* induce the least dispersal *ex vivo*. Moreover, we find that generic glycoside hydrolases, which previous work has shown to be highly effective at inducing dispersal *in vivo* and *ex vivo*, have no measurable effect on the mechanics of biofilms grown *in vitro*. This highlights the possibility that effective approaches to eradicating biofilms may depend strongly on the growth environment.

Introduction

Bacterial biofilms consist of bacteria embedded in a matrix of extracellular polymers and proteins. Bacteria make multiple types of matrix polymers, and the importance of a specific polymer type varies with biofilm-forming species and by strain within a species. For biofilm infections, the matrix protects the biofilm bacteria chemically by inhibiting the diffusion of antibiotics into the biofilm and by binding to antibacterial chemicals produced by the host immune response.[1-3] Furthermore, the mechanical

integrity and structure of the bacterial biofilm conferred by the matrix gives rise to stable microenvironments that contribute to phenotypic antibiotic tolerance.[4-5] *Pseudomonas aeruginosa* is an opportunistic human pathogen, known for its ability to form robust biofilms.[6-7] Many common interventions in *P. aeruginosa* infections, like antibiotic treatment, are far more successful if the bacteria are in a planktonic state rather than in a biofilm.[8] It has been shown that dispersing biofilms into single cells or smaller aggregates can increase the efficacy of antibiotics and of phagocytic immune cells to against bacteria.[8-11] However, in cases where the patient already has a well-established biofilm infection, finding methods to mechanically compromise and disrupt biofilms without harming the patient is often non-trivial. *P. aeruginosa* biofilms have proven mechanically resilient against many simple perturbations such as ionic disruption, pH changes, and addition of small organic molecules, hinting that once the matrix network has formed, connections are difficult to break.[12] Therefore, more and better methods for disrupting established biofilms are needed.

The glycoside hydrolases cellulase and α-amylase both break down polysaccharides by hydrolysis of glycosidic linkages; cellulase hydrolyzes β-1,4 bonds and α-amylase hydrolyzes α-1,4 bonds. Although cellulase and α-amylase specifically attack cellulose and amylose, respectively, they can also break down other, structurally-similar polysaccharides.[11] Both cellulase and α-amylase can inhibit *P. aeruginosa* biofilm growth, and they can cause the dispersal of bacteria from biofilms grown in wounds, rendering bacteria more susceptible to antibiotics.[10-11, 13-14] However, neither cellulase nor α-amylase are specific against any components of the *P. aeruginosa* biofilm matrix.

It is plausible that specific enzymes might cause dispersal with greater efficiency than generic glycoside hydrolases, with less potential for harming the host.

Biofilms formed by different strains of *P. aeruginosa* can be dominated by different types of extracellular polymers (EPS). We expect this to determine how biofilms respond differently to enzymes that attack specific polymers. The primary EPS constituents of *P. aeruginosa* biofilm matrices are the polysaccharides alginate, Psl, and Pel, and a non-polysaccharide, extracellular DNA (eDNA).[15-16] The enzymes alginate lyase (which targets glycosidic linkages, breaking alginate down into smaller oligosaccharides via a β-elimination reaction at β-1,4 bonds[17]) and DNase (which cleaves phosphodiester bonds in the backbone of DNA *via* hydrolysis, breaking it down into pieces as small as 10 base pairs) have successfully been used *in vitro* to disrupt the structure of *P. aeruginosa* biofilms, rendering them more susceptible to the immune system and antibiotics.[9, 18-23] Similarly, Psl- and Pel-specific glycoside hydrolases increase antibiotic susceptibility and decrease biomass when they are used to treat Psl- and Pel-dominant biofilms, respectively.[24] However, how different EPS-specific enzymes impact biofilm mechanics remains unknown. This gap in understanding hinders the development of strategies that use enzymes to specifically weaken biofilms to facilitate clearance.

In this study, we grew biofilms *in vitro* from lab strains of *P. aeruginosa* that each produce primarily only one extracellular polysaccharide, alginate, Psl, or Pel, and treated biofilms with enzymes that are specific to alginate and eDNA. We used bulk oscillatory rheology to quantify the changes in biofilm mechanics resulting from enzymatic treatment. We expected to find that the effect of treatment would be greatest when the enzyme was matched to a dominant matrix polymer. Indeed, alginate lyase has its biggest effect on

the mechanics of alginate-dominant biofilms and DNase I has its biggest effect on the mechanics of Pel-dominant biofilms; Pel has been shown to bind to eDNA in the matrix.[25] Biofilms that have had their matrix networks compromised by specific enzymatic treatment also have increased diffusive transport of water. Upon treatment with the glycoside hydrolases cellulase and α-amylase, which are not specific to matrix polymers in the *in vitro* biofilms we study, the mechanical alterations are small and not statistically significant.

We had expected these results to translate to the dispersal of biofilms *in vivo*. However, for biofilms grown *in vivo*, in a mouse model of wound infection, and then excised and treated *ex vivo*, the generic glycoside hydrolases are more effective than the specific enzymes at inducing dispersal (*i.e.,* transition of the bacteria from a biofilm to a planktonic state) regardless of the lab strain of bacteria used. Moreover, and to us more surprisingly, the biofilms that had the greatest dispersal response when treated *ex vivo* were those grown by the lab strain for which *in vitro* enzymatic treatments had the least effect. This could be because the matrix composition of biofilms grown *in vivo* might be significantly different from that of biofilms grown *in vitro* by genetically-identical strains of bacteria. If so, this highlights the importance of the biofilm growth environment and the need for taking growth conditions into account when devising anti-biofilm strategies. This might also be because the dispersal of constituent bacteria may not depend primarily on the mechanics of the embedding biofilm matrix, and may indicate the need for better understanding of the mechanisms underlying dispersal.

Methods

Bacterial Strains and Growth Conditions

The primary bacterial strains used for rheological studies and SEM are all in the *P. aeruginosa* background: PA01 wild-type (WT) (which makes Psl and Pel but only small amounts of alginate *in vitro*), PA01 Δ*wspF* Δ*pel* (which over-expresses Psl), PA01 Δ*wspF* Δ*psl* (which over-expresses Pel), and PA01 Δ*mucA* (which over-expresses alginate). [26-30] Each of these strains constitutively expresses green fluorescent protein, so that any future experiments with fluorescent microscopy may be done with the same strain of bacteria.

To complement our findings in the PA01 background and test their generality, we also used other strains of *P. aeruginosa*. PA14 WT is a lab strain that makes Pel but not Psl [31]. Clinical strains, isolated at different timepoints from two different patients with cystic fibrosis, give us two parallel evolutionary histories, each beginning with an ancestor and continuing with two descendent strains that were isolated at the same time; one descendent has increased alginate production and the other does not [32].

Prior to beginning biofilm growth, we streaked frozen bacterial stock on an luria broth (LB) agar plate, picked one colony after overnight growth, and used that colony to initiate growth in liquid culture, which we grew shaking in 4mL luria broth (LB) liquid media overnight at 37°C.

Application of Biofilm Treatments for Rheology

To grow enough biofilm for rheological study, we spread 250μL of overnight growth on LB agar plates of standard size, 100mm x 15mm, and let these grow overnight at 37°C. Once the biofilms have grown overnight on LB agar plates, we apply our biofilm

treatments by adding a liquid layer of treatment solution, 50μL to 100μL in volume, to the top of the lawn of biofilm on the plate. We use an L-spreader to gently—with minimal disruption to the biofilm—spread the liquid evenly over the biofilm. The treatments used in this experiment are Optizyme DNase I (Fisher), alginate lyase (Sigma), α-amylase from *Bacillus subtilis* (MP Biomedicals), cellulase from *Aspergillis niger* (MP Biomedicals), L-arginine hydrochloride (Fisher), and Salmon Sperm DNA (Fisher). For each treatment, we also treat with the same volume of solvent without enzyme as a control. The DNase I enzyme is buffered in 100mM Tris-HCl (7.5pH), 25mM $MgCl_2$, and 1mM $CaCl_2$, so the control for the DNase I treatments was this buffer solution alone. All other enzymes were in de-ionized (DI) water, and the corresponding control treatment was DI water. The treatment and control is then left to sit upright for an hour at 37°C. After an hour, the biofilm plate is ready for rheological measurement. Concentrations for the enzyme treatments are reported in activity units (U) per milliliter, where 1 U is the amount of enzyme that catalyzes one micro-mole of substrate per minute. This was determined based on information from the supplier.

Rheological Measurement

Rheology is done similarly as previously described in Kovach *et al.*, with minor geometry changes.[33] For this study, we used a stress-controlled AR 2000ex rheometer with a parallel-plate geometry with 8mm steel head. The biofilm is gently scraped from the surface of the agar onto the bottom plate of the rheometer; this typically only takes one plate of biofilm to fill the gap. The rheometer head is then lowered to a 500μm gap. Excess biofilm was trimmed to appropriately fill the gap; this takes approximately 60μL of

biofilm. The rheological measurements were run at room temperature with the same solvent trap from Kovach *et al.* to stop evaporation of water from the biofilm while it was on the rheometer. [33]

For each sample, we first run a frequency sweep from 0.1 to 200 rad/s at 1% strain to test the frequency dependence of the biofilm mechanics. In general, the biofilms have low frequency dependence from approximately 0.1 to 100 rad/s. After the frequency sweep, we run a strain sweep at 3.14 rad/s from 0.1 to 1000% strain.

Analysis of Rheological Data

Storage modulus and yield strain were determined from the strain sweep data. Storage modulus is determined to be the value of G' in the plateau region of the strain sweep – *i.e.*, in the linear viscoelasticity regime. To determine yield strain, we fit the plateau region to a linear fit and fit the nonlinear region to a power law. We report the intersection of these fits as the yield strain. The yield stress corresponding to this yield strain is then taken from the raw rheological data.

Biofilm Drying Measurements

We grow biofilms as we do for rheology. We spread 250μL from liquid overnight onto LB agar plates of standard size, 100mm x 15mm, and let these grow overnight at 37°C. The biofilm is then gently scraped from the plate onto a weigh boat. For Δ*mucA* (Alg+) biofilms, we add 100μL of treatment such that the concentration is 200 U/mL alginate lyase (Sigma) in deionized water and for Δ*wspF* Δ*psl* (Pel+) biofilms, we add 100μL of treatment such that the concentration is 500 U/mL deoxyribonuclease I from

bovine pancreas (Sigma) in 0.15M NaCl. The weigh boat with biofilm and treatment is then weighed. The biofilm is moved from the weigh boat onto a clean LB agar plate, which acts as a sink for the excess water in the biofilm. This parallels treatment conditions for our rheology measurements, and we found that this gave rise to faster drying than allowing the biofilm to dry on a weigh boat without an agar sink. This plate is then moved to an incubator at 37°C for one hour. After the hour, the biofilm is returned to its weigh boat and weighed. The alteration in weight is then calculated as water loss by $\%Water\ Loss = \frac{Hydrated\ weight - Dried\ Weight}{Hydrated\ Weight} \times 100$.

Statistics

All rheological, drying, and dispersal measurements were done in triplicate – *i.e.* with three independent replicates for both treatment and control - except for a few cases in which six independent replicates were used, as indicated in the corresponding figure captions. Testing for statistical significance using a two-tailed T-test was done in Microsoft Excel.

Scanning Electron Microscopy (SEM)

To image biofilms in SEM, we grow the biofilms on small glass pieces, 6mm x 1cm, which have been cut from standard microscope slides. We place these glass pieces into the wells of a 24 well plate. For growth of biofilm, we add 10μL of overnight growth to 1mL of LB in the wells. The biofilm is left to grow on the glass pieces for 24 hours as a static culture. At 24 hours, we gently pull the supernatant from the wells, leaving the biofilm growth on the glass pieces intact, and add the enzyme treatment to the biofilm. We add

500μL of treatment and controls to the wells. For SEM, we use alginate lyase (Sigma) in deionized water and deoxyribonuclease I from bovine pancreas (Sigma) in 0.15M NaCl. The alginate lyase treatment is at a concentration of 200U/mL and the DNase I treatment is 500U/mL. The treatment and the controls are let to sit for one hour at 37°C. After one hour, we wash the treatment out gently with PBS twice.

For standard fixation steps for SEM, we then move the glass pieces into a new 24 well plate. The first step is fixation with 1mL 4% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer and 2mM Ca^{2+} and 4mM Mg^{2+}; this step causes proteins in the sample to irreversibly cross-link. The samples are left in the aldehyde solution overnight at room temperature. After overnight aldehyde fixation, the aldehyde is washed from the samples with 0.1M cacodylate buffer 3 times with 10 minutes between each wash. We then stain the samples with a mix of 4% osmium tetroxide and 4% potassium ferrocyanide (OsFeCN) at 1:1 ratio, giving us a solution of reduced osmium; the staining is set by microwaving at vacuum at 100W for 2 minutes twice, with 2 minutes of wait time between. Reduced osmium crosslinks with the lipids in the membrane of the bacterial cells, increasing membrane contrast to electrons for imaging. After the osmium fixation, we wash the osmium solution from the wells of the plate with deionized water. Once the osmium solution has been removed, we dry the sample.

The first step in drying is replacing the water in the sample with ethanol by placing the samples in 50%, 75%, and 95% ethanol for 10 minutes each sequentially. Then the sample sits in 100% ethanol for 10 minutes twice. We then dry the samples with a critical point drier. Once the sample is dried, it is fixed to SEM mounts using carbon tape and grounded with colloidal graphite paint around the edges of the sample. We then sputter

coat the sample with 14nm platinum/palladium. For imaging, we use a ZeissSupra40 Scanning Electron Microscope operated at 5keV. The detector used captures type II secondary electrons. These electrons scatter at a wide angle and therefore capture compositional and some topographical information of the surface due to larger penetration depth, as opposed to type I secondary electrons that scatter at a smaller angle and are more sensitive to topological information.

Murine Chronic Wound Model

Strains of *P. aeruginosa* were grown in baffled Erlenmeyer flasks at 200rpm in LB at 37°C, from which planktonic cells were harvested for injection into the wound. A full description of the chronic wound model used in this study can be found in previous work.[11, 34-37] Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbital. After a surgical plane of anesthesia was reached, the backs were shaved and administered a full-thickness, dorsal, 1.5 x 1.5 cm excisional skin wound to the level of panniculus muscle with surgical scissors. Wounds were then covered with a semipermeable polyurethane dressing (OPSITE dressing; Smith & Nephew®), under which 10^4 bacterial cells were injected into the wound-bed. Biofilm formation was allowed to proceed for 72 hours, after which the mice were euthanized, and the wound-beds were harvested for *ex vivo* treatment with vehicle control, Alginate Lyase + DNAse, or Alpha-Amylase + Cellulase. Colony forming units (CFUs) were determined via serial dilution plating on *Pseudomonas* isolation agar, and percent bacterial cell dispersal was calculated by finding the quotient of the total CFU (biofilm-associated plus planktonic) divided by the planktonic CFUs (in the supernatant). Animals were treated humanely and

in accordance with protocol #07044 approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center in Lubbock, Texas.

Results and Discussion

Enzymatic treatments were applied in parallel with control treatments. To minimize the effects of variations in culturing conditions such as humidity on the day of growth, the biofilms for each pair of treatment and control were initiated from the same overnight liquid culture and grown in the same incubator, on nutrient agar plates from the same preparation batch. For each pair of treatment and control biofilm, rheological measurements were performed on the same day, in immediate succession. Examples are shown for alginate lyase treatment in Figure 1. To determine the effects of the treatments on different mechanical properties, we report a ratio of the value of a mechanical property (storage modulus, yield strain, or yield stress) for a treated biofilm to the value of the same property for the corresponding control biofilm. This is very similar to the approach we took in our earlier work, and is intended to account for the effects of day-to-day variation in the measured mechanics of biofilms grown from the same bacterial strain.[33] Because these biofilms are primarily solid-like, with storage moduli approximately an order of magnitude greater than viscous moduli[33], in this study we focus exclusively on solid-like mechanical properties, namely the storage modulus G´, yield strain, and yield stress, which for a dominantly solid-like material is approximately the product of G´ and yield strain.

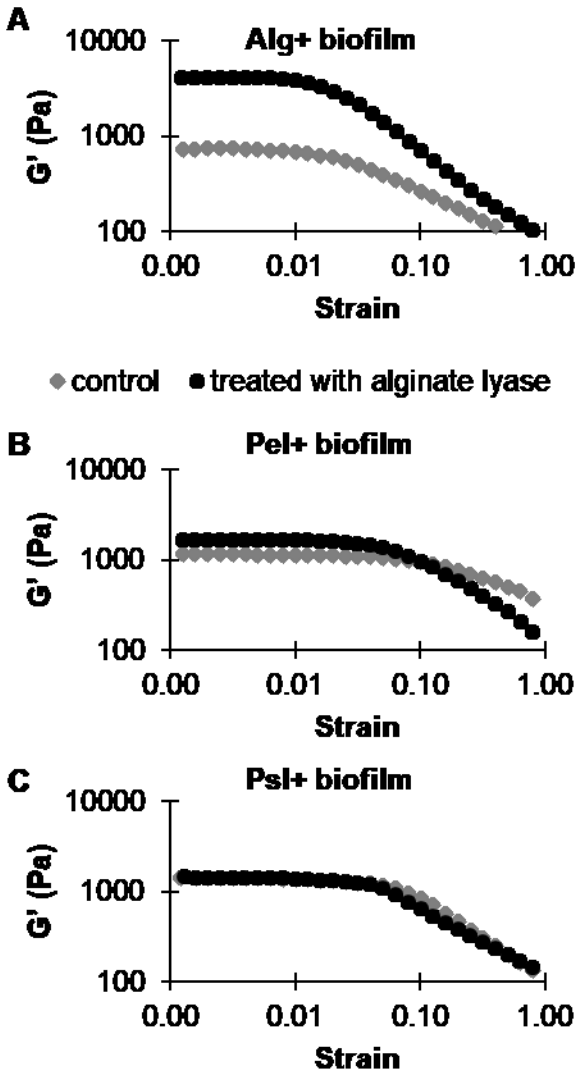

Figure 1. Sample rheological data comparing control biofilms with those treated with alginate lyase. For the sake of clarity, because this work focuses on solid-like properties, only the storage modulus G' is shown. (A) The alginate-dominated biofilm shows the biggest shift in mechanics, compared with its control. (B) The Pel-dominated biofilm shows a much smaller shift in mechanics, compared with its control. (C) The Psl-dominated biofilm shows no appreciable shift in mechanics, compared with its control.

Bacterial strains used

To study the response of different polymer types to each enzyme, we use four PA01 lab strains of *P. aeruginosa*, three of which have been genetically modified to alter polysaccharide production. *In vitro*, wild type (WT) PA01 produces primarily Pel and Psl, and does not produce significant amounts of alginate.[38] To examine the response of Pel and Psl, we use the strains Δ*wspF*Δ*psl* and Δ*wspF*Δ*pel*, respectively. The deletion of the *wspF* gene causes overproduction of cyclic-di-GMP, an intracellular signaling molecule that increases the constitutive expression of both Pel and Psl.[33, 39] Deleting either the *pel* or *psl* gene in addition to *wspF* forces overproduction of the remaining polysaccharide. In the figures, Δ*wspF*Δ*psl* is denoted as Pel+, and Δ*wspF*Δ*pel* is denoted as Psl+. To characterize the response of alginate, we use PA01 Δ*mucA*.[33] Disrupting the function of the *mucA* gene results in the overproduction of alginate. Although Pel and Psl are present in the Δ*mucA* biofilm, we have previously shown that alginate has a strong influence on mechanical properties for Δ*mucA* biofilms.[33] In the figures, Δ*mucA* is denoted as Alg+.

To test the extent to which our results for the PA01 background may be generalizable to other *P. aeruginosa* strains, we use PA14 (WT), which produces Pel and not Psl, and clinical strains chronologically isolated from two cystic fibrosis patients denoted A and B. Clinical strains include two ancestor strains, A1 and B1, two descendant strains that independently evolved to have increased alginate production, A3.1 and B3.1, and two descendant strains that evolved from the same ancestor and were isolated at the same timepoint but have largely unchanged alginate production, A3.2 and B3.2.[32]

Alginate lyase and DNase I act on biofilm mechanics with specificity

We find that treating Psl+, Pel+, and Alg+ biofilms with 200 U/mL alginate lyase causes statistically-significant changes in the mechanical properties of both Pel+ and Alg+ biofilms (Figure 2 A and B), but not Psl+ biofilms (Figure 2 C). The effect on the storage modulus and yield stress of the Alg+ biofilm is greater, as both increase by more than a factor of five. In contrast, the storage modulus of the Pel+ biofilm increases by a factor of less than 1.5, and its yield stress actually decreases due to the treatment causing a decrease in the yield strain (Figure 2). Alginate lyase decreases the yield strain of Alg+ and Pel+ biofilms similarly, by ~40% and ~45% respectively.

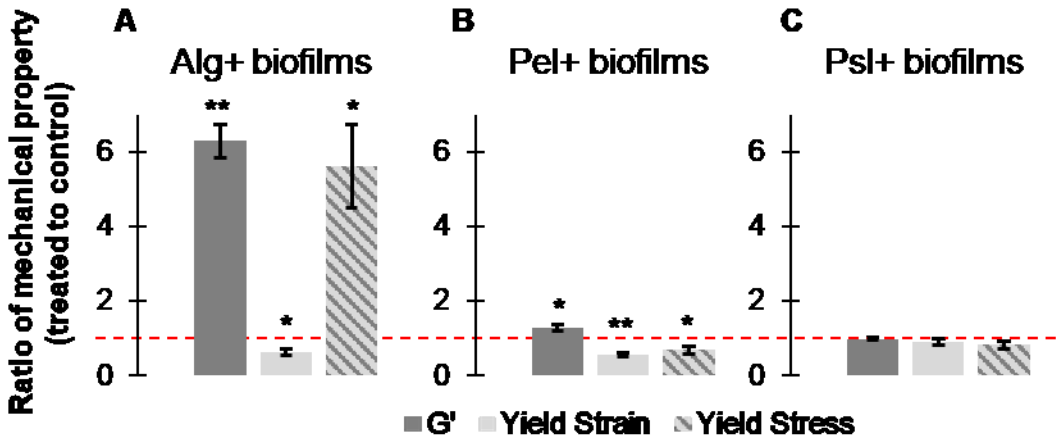

Figure 2. Treatment by 200 U/mL alginate lyase impacts the mechanics of (A) alginate-dominant biofilms and (B) Pel-dominant biofilms; there is no statistically-significant effect on the mechanics of (C) Psl-dominant biofilms. G', yield strain, and yield stress values measured for a treated biofilm are compared to G', yield strain, and yield stress values measured for a biofilm treated by the control solution by taking ratios of treated to control values. Thus, a ratio of one (indicated by the red dashed line extending across all three panels) indicates no change upon enzyme treatment, a ratio greater than one indicates

an increase upon treatment, and a ratio less than one indicates a decrease upon treatment. * p ≤0.05 and ** p≤0.01 by a Student two-tailed T-test, with the null hypothesis being no effect of the enzyme, *i.e.* the ratio is unity. Error bars are standard error of the mean. N=3 for each measurement.

When biofilms are treated with 500 U/mL DNase I, we find that the storage modulus of Pel+ biofilms, and no other biofilm type, increases by more than a factor of two (Figure 3 A). This parallels the effect of alginate lyase on the storage modulus of Alg+ biofilms. Pel is thought to associate with eDNA in the matrix[25], and our mechanical measurements are consistent with the idea that Pel and eDNA interact to mechanically stabilize these biofilms. DNase I decreases the yield strain of Pel+ biofilms by ~35% (Figure 3). For both alginate lyase treatment of Alg+ biofilms and DNase I treatment of Pel+ biofilms, the decrease in yield strain likely occurs due to the chains of the polymer network being shortened, so that that the matrix cannot maintain integrity in the face of large deformations.

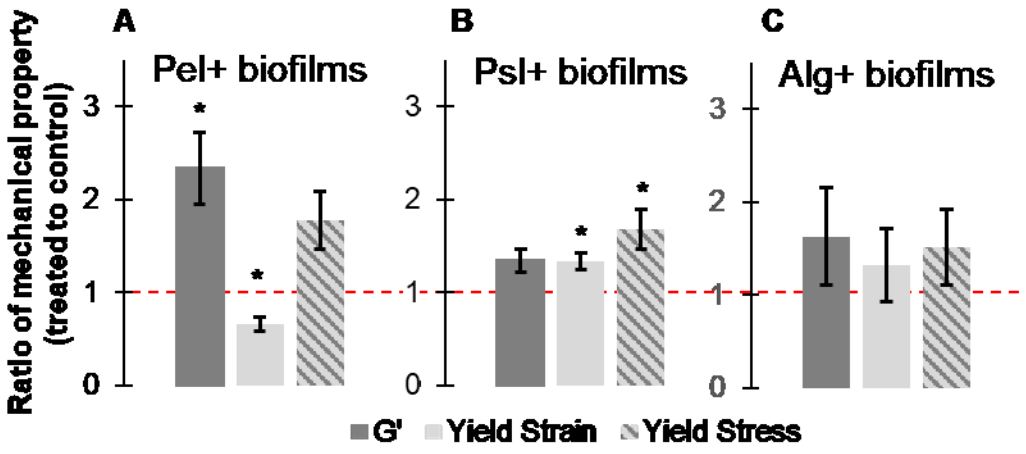

Figure 3 Treatment by 500 U/mL DNase I impacts the mechanics of (A) Pel-dominant biofilms and (B) Psl-dominant biofilms but not (C) alginate-dominant biofilms. * $p \leq 0.05$ and ** $p \leq 0.01$ from a Student two-tailed T-test, with the null hypothesis being that there is no effect of the enzyme – *i.e.*, that the ratio is unity, indicated by the red dashed line. Error bars are standard error of the mean. N=3 for each measurement in Panels A and B; N=6 for each measurement in Panel C.

Despite the effect of alginate lyase on the Pel+ biofilms, it seems unlikely that alginate lyase is catalytically active on the Pel polysaccharide. Pel and alginate are composed of different monomer units; Pel is composed of N-acetylglucosamine and N-acetylgalactosamine, and alginate is composed of mannuronate and guluronate.[40-42] They also have opposite charges, with Pel being cationic and alginate being anionic. While the Pel+ biofilm should only have minimal alginate present, it is possible that the small amount present interacts electrostatically with the Pel polysaccharide network in such a way as to increase yield strain, as we have previously suggested may happen for anionic eDNA binding electrostatically to Pel in the biofilm matrix.[33] We address why cleaving matrix polymers results in a higher measured storage modulus below, in the subsection "*The increase in storage modulus is an effect of drying*."

In previous studies by others, the dispersal properties of alginate lyase were found to be catalysis-independent; merely the presence of any protein triggered a generic dispersal signal in *P. aeruginosa*.[43] However, the strongly specific activity of alginate lyase on Alg+ biofilms that we measure in our experiments indicates that the enzyme is specifically cleaving the alginate. As a further check to confirm the importance of

363 enzymatic specificity in this case, we treat an Alg+ biofilm with arginine, a main amino
364 acid component of alginate lyase that has been found to trigger dispersal.[43] Treatment
365 with arginine had no effect on the mechanics of the Alg+ biofilm (Figure S1). Treatment
366 with arginine had some effect on the WT PA01 biofilm—with matrix composed primarily
367 of Psl and secondarily of Pel—but the mechanical alteration did not mirror that of alginate
368 lyase (Figure S2). This is additional evidence that the changes we measure in biofilm
369 mechanics are specific to the effect of alginate lyase on alginate in the matrix.
370 DNase I does cause a statistically-significant, but small, increase in the yield strain
371 and yield stress of Psl+ biofilms (Figure 3 B). The mechanism underlying this result is
372 less clear, but it likely reflects the smaller, but still present, role of eDNA as a structural
373 constituent in these biofilms.[16, 44-45] DNase I has no statistically-significant impact on Alg+
374 biofilms (Figure 3 C).
375
376 *Mechanical alterations are dose-dependent*
377 To probe how mechanical changes depend on the amount of polymer cleavage,
378 we vary the enzyme concentrations. At lower enzyme concentrations, the only
379 statistically-significant mechanical change is a ~130% increase in storage modulus when
380 we treat an Alg+ biofilm with 20 U/mL alginate lyase; this is much less than the ~530%
381 increase in storage modulus when we treat with 200 U/mL alginate lyase (Figure 4 A).
382 Similarly for DNase I, treating Pel+ biofilms with an enzyme concentration of 500 U/mL
383 has statistically-significant effects, but a concentration of 50 U/mL does not (Figure 4 B).

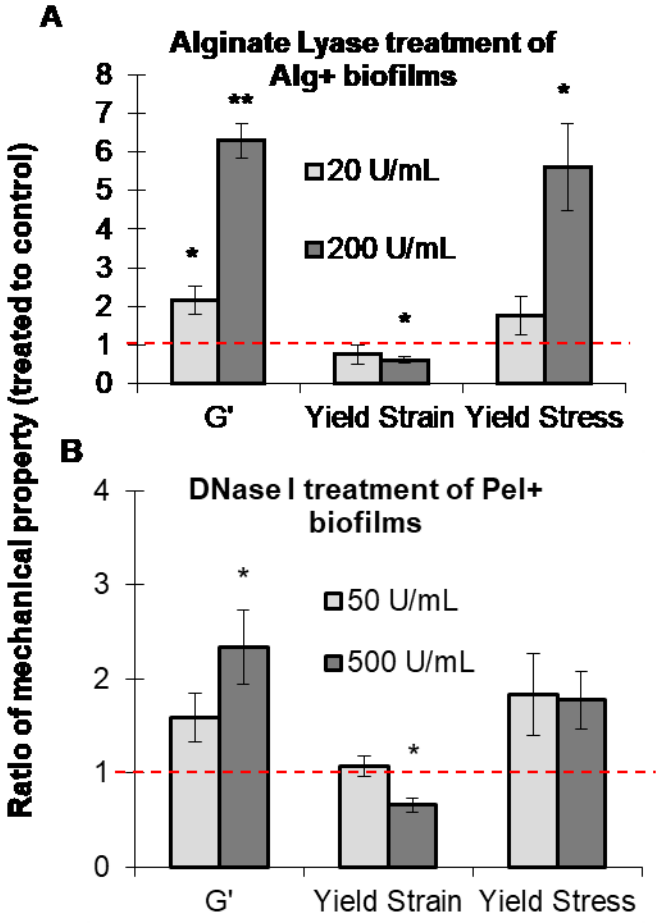

Figure 4. The effects of enzyme treatment is dosage-dependent, for (A) alginate lyase treatment on alginate-dominant biofilms and (B) DNase I treatment on Pel-dominant biofilms dosage. * $p \leq 0.05$ and ** $p \leq 0.01$ from a Student two-tailed T-test, with the null hypothesis being that there is no effect of the enzyme – *i.e.*, that the ratio is unity, indicated by the red dashed line. Error bars are standard error of the mean. N=3 for each measurement.

Specific disruption of other P. aeruginosa strains

To test the degree to which the results described above for variants of the lab strain PA01 may be extensible to other strains of *P. aeruginosa*, we apply the same techniques

to biofilms grown from the lab strain PA14 (WT), and from two groups of chronological clinical isolates.

PA14 makes Pel but not Psl nor significant amounts of alginate, and its biofilms are considered Pel-dominant [31]; therefore, we expect its response to enzymatic treatment to resemble that of Pel+ biofilms. We find that treatment of wild-type PA14 biofilms with 200 U/mL alginate lyase or with 500 U/mL DNAse I both cause statistically-significant increases in yield stress of ~340% and ~150%, respectively (Figure S3), but no statistically-significant changes in elastic modulus or yield strain. These are unlike the results we found upon treating Pel+ biofilms (Figures 2B and 3C). However, the change upon treatment with DNAse I is more statistically significant than the change upon treatment with alginate lyase, which is congruent with the results seen for the Pel+ biofilms.

How Pel production differs between clinical isolates of *P. aeruginosa* has not, to our knowledge, been measured, but it has long been known that *P. aeruginosa* biofilm infections in the lungs of cystic fibrosis patients often evolve to increase the production of alginate and that this is associated with worse outcomes for patients [46]. Therefore, to investigate how our findings for PA01 might apply to clinical strains, we treat biofilms grown from clinical strains with alginate lyase. Upon treatment, we find no statistically-significant changes in the mechanics of the ancestor strains, A1 and B1 (Figures S4 A and S5 A, respectively); these ancestor strains do not have high levels of alginate production. We do measure statistically-significant changes in yield strain for descendant strains with increased levels of alginate production (Figures S4 B and S5 B), although the direction of the shift in yield strain upon treatment is not consistent. As a check for how

418 the response to enzyme treatment might be impacted by evolutionary changes not
419 associated with increased alginate production, we also used alginate lyase to treat strains
420 that were descended from the same ancestor and isolated at the same timepoint as the
421 high-alginate descendents but that did not have high alginate production. For one such
422 low-alginate descendent we found no significant change in elasticity, yield strain, or yield
423 stress upon treatment (Figures S4 C). For the other we found a decrease in elasticity at
424 a lower level of statistical significance than the changes in yield strain found when high-
425 alginate descendent strains were treated with alginate lyase (Figure S5 C).

426 Any causative interpretation of these results (for both PA14 and the clinical strains)
427 in terms of molecular interaction between enzymes and matrix components is less
428 straightforward than it is for the over-expressing strains in the PA01 lab background
429 studied above. In particular, further characterization of the matrix components of the
430 clinical strains would greatly benefit future understanding. However, we do see that more
431 statistically-significant changes are found, and with a better level of statistical significance,
432 when the treating enzyme is matched to a dominant component of the biofilm matrix. This
433 broadly agrees with our findings for PA01 strains.

434

435 *Electron microscopy suggests specific disruption of matrix structure*

436 To gain some qualitative insight into the microstructural changes associated with
437 specific enzyme treatment, we use scanning electron microscopy (SEM) to image Alg+
438 and Psl+ biofilms, both untreated and after treatment with alginate lyase (Figures S6 and
439 S7), and Pel+ and Psl+ biofilms, both untreated and after treatment with DNase I (Figures
440 5 and S8). Most dramatically, we see that an untreated Pel+ biofilm has 100-micron scale

structures (Figure 5C) and that the cells are embedded in an interconnected network of stringy polysaccharides (Figure 5D). When treated with DNase I, these large structures and surface attachment of cells are compromised (Figure 5A), and there are less network strands present (Figure 5B). It appears that the loss of yield strain in Pel+ biofilms due to treatment by DNase I is most likely due to the reliance of the Pel network on extracellular DNA for its high yield strain when untreated.

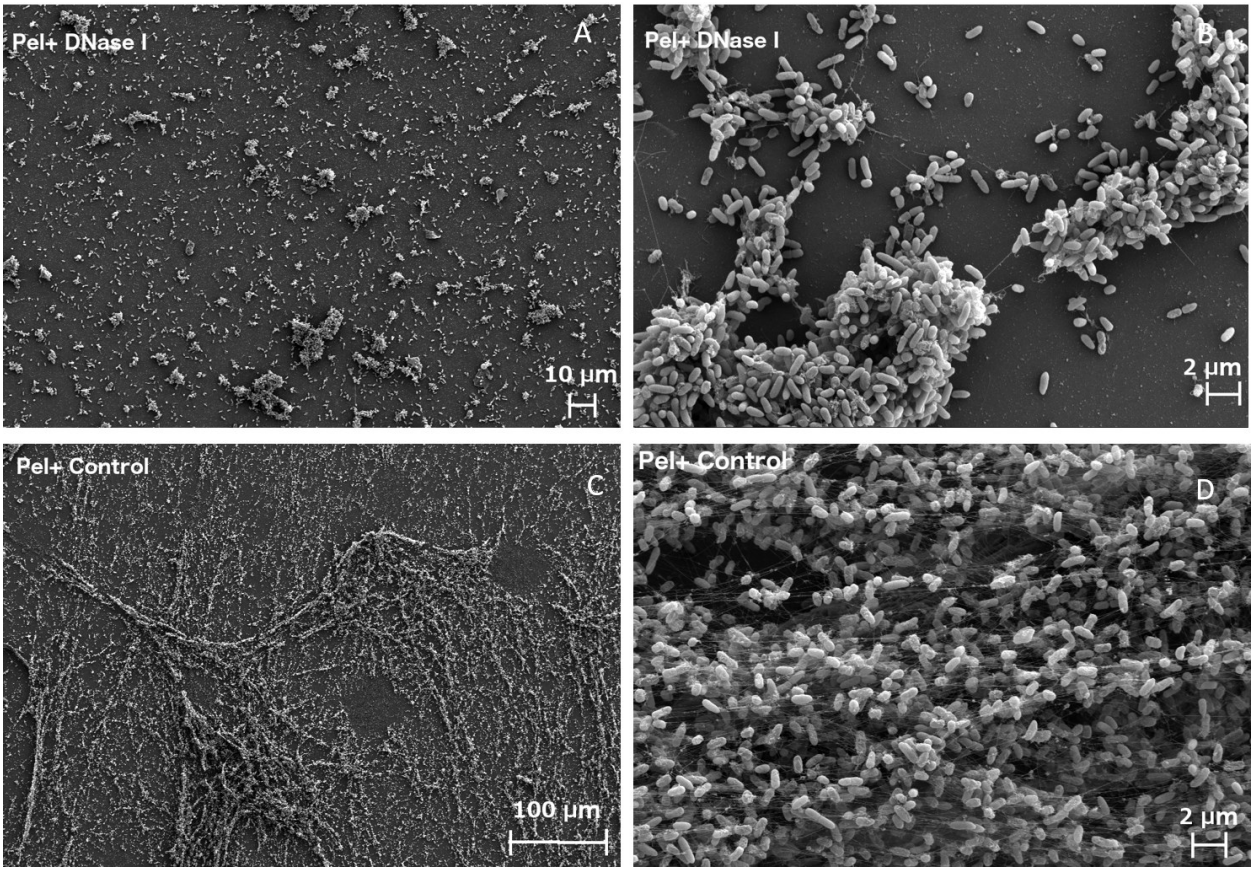

Figure 5. SEM images of Pel-dominant biofilms. (A, B) Biofilms that have been treated by DNase I show (A) no long-range interconnectivity and (B) no polymer fiber strands. In

contrast, control biofilms that were not treated by DNAse I have (C) long-range interconnectivity and structure and (B) visible polymer fibers bridging bacteria.

Despite alginate lyase causing mechanical changes in Alg+ biofilms, there is not a dramatic difference in the SEM images of treated and untreated biofilm (Figure S6). Preparing samples for SEM imaging causes violent agitation to the biofilm formations on the surface. Alg+ biofilms are less cohesive than WT, Psl+, or Pel+, even without any treatment.[33] Therefore, we may be selectively imaging only the strongest attachments and that may be a reason that we do not observe changes in structure upon enzyme treatment of Alg+ biofilms. Furthermore, simulations of enzymatic treatment on biofilms have shown that if the EPS network does not contribute to cohesiveness, surface growth is difficult to remove.[47] Thus, an Alg+ biofilm may be difficult to alter at the surface.

We also imaged the effects of enzyme treatments on Psl+ biofilm, for which they do not cause significant mechanical compromise. There may be some alteration in surface cell-density in treatment with DNase I on Psl+ biofilm (Figure S8). We speculate that, although extracellular DNA does not play a major role in the mechanical properties of Psl+ biofilms, it may nevertheless play a role in cellular attachment to the surface. Alginate lyase also appears to slightly affect Psl+ biofilm surface attachment (Figure S7 A and S7 C) as well as alter the visible polysaccharide network (Figure S7 B and S7 D). As there may be small amounts of alginate present in our Psl+ biofilms, it may be that these alginate polysaccharides do play some role in surface attachment, while not being mechanically important to the bulk matrix.

475 *The increase in storage modulus is an effect of drying*

476 Unlike the decrease in yield strain, it is not immediately obvious why enzymatic
477 treatment, resulting in shorter polymers, should increase storage modulus. Of greater
478 pragmatic concern, an increase in storage modulus (which could also cause an increase
479 in yield stress as well, as seen in Figure 2A), would seem likely to be protective for the
480 bacteria in the biofilm, and therefore not a desirable outcome if the specific enzymes
481 alginate lyase and DNase are to be considered as an approach to clinical treatment.

482 However, the interpretation of these *in vitro* experiments is more complex than it
483 may at first appear. A modified version of Fick's first law of diffusion gives us the drying
484 of a polymer gel or network related to the diffusion coefficient of the solvent by

485 $$N = -\rho D_{eff} \frac{dX}{dZ},$$

486 where N is the drying rate, ρ is the volume density of polymer, D_{eff} is the effective diffusion
487 coefficient of the solvent, X is the solvent content, and Z is the thickness of the matrix.[48]
488 By cleaving polymer chains, enzymes reduce the connectivity of the biofilm matrix and
489 therefore allow water to diffuse more freely through the matrix.

490 In order to verify that the biofilms are drying, biofilms are weighed before and after
491 treatment with enzymes and a control. When an Alg+ biofilm is treated with alginate lyase,
492 it dries ~50% more than a biofilm treated with a control (Figure 6). When a Pel+ biofilm is
493 treated with DNase I, it dries ~20% more than the control (Figure 6). Visual inspection of
494 Alg+ biofilms also shows a drier surface for treated than control biofilms (Figure S9).
495

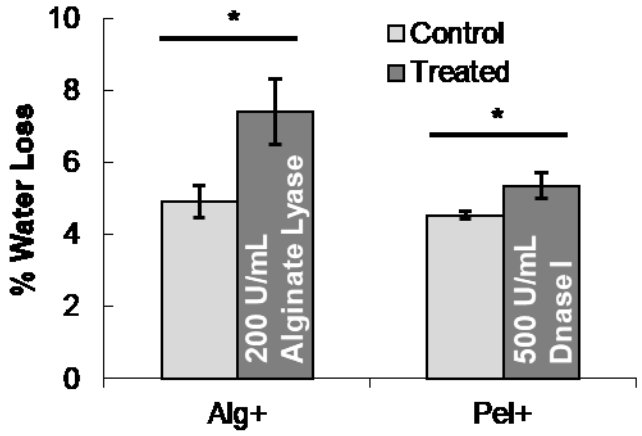

Figure 6. Biofilms that have been treated with enzymes dry more quickly than control biofilms. When alginate-dominant biofilms are treated with 200 U/mL alginate lyase, on average they lose 7.4% of their weight to water loss, while the control biofilms lose only 5%. When Pel-dominant biofilms are treated with DNase I, on average they lose 4.5% of their weight to water loss, while the control biofilms lose only 5.4%. * $p \leq 0.05$ from a Student two-tailed T-test, with the null hypothesis being that the water loss is the same for the treated and untreated biofilm. Error bars are standard error of the mean. N=3 for each measurement.

Thus, during the hour of enzyme treatment at 37°C, enzyme-treated biofilms dry more quickly than do biofilms treated with an inactive control. Therefore, when biofilms are measured in the rheometer, the enzymatically-compromised biofilms contain less water and therefore have a higher polymer concentration than do the corresponding control biofilms. It is well known that elasticity scales with polymer density $G' \propto c^A$, where c is polymer concentration and A is a scaling factor (A=2.25 for an entangled polymer in a good solvent).[49] Thus, the increase in storage modulus most likely reveals a marked

drop in polymer moisture content due to the polymer losing the ability to hold water, rather than any increase in structural cohesiveness or integrity.

It is important to note that for biofilms infecting the body, or for any other condition where there is surrounding fluid or high humidity, increased diffusion would **not** be expected to result in drying. However, transport of antibiotics, immune factors, and other chemicals would be increased.

Glycoside hydrolases cellulase and α-amylase only minimally impact biofilm mechanics

Our results above suggest that the efficacy of enzymes in altering biofilm mechanics is likely to strongly depend on enzyme-specific activity against a dominant matrix constituent. However, it has been found that cellulase and α-amylase, which are not specific to any matrix component produced by the biofilm bacteria, are successful in inhibiting and dispersing *P. aeruginosa* biofilms grown *in vivo*, in wounds, into a planktonic-like acute infection state.[11] It seems likely that dispersal should be associated with mechanical compromise of the biofilm. Therefore, we tested the effect of both cellulase and α-amylase on the mechanics of Psl+, Pel+, and Alg+ biofilms.

Upon treatment with 5% α-amylase, Psl+ biofilms experience a ~5% decrease in storage modulus, and Alg+ biofilms experience a ~40% increase in yield strain; no other statistically-significant effects are seen (Figure S10). Upon treatment with 5% cellulase, the yield stress of Alg+ biofilms decreases by ~60%; no other statistically-significant effects are seen (Figure S11). Thus, compared with the more than 500% increase in storage modulus and yield stress experienced by Alg+ biofilms upon treatment with alginate lyase, and the more than 200% increase in storage modulus experienced by Pel+

biofilms upon treatment with DNase I (Figures 2 and 3), the effects of non-specific glycoside hydrolases on biofilm mechanics are negligibly small. It is notable that the increase in yield strain experienced by Alg+ biofilms treated by α-amylase parallels, for our results with specific enzymes, only the increase in yield strain seen for Psl+ biofilms treated by DNase I, and that we do not expect specific activity of DNase I against the Psl extracellular polysaccharide.

Previous work using generic glycoside hydrolases to disperse wound biofilms used WT PA01.[11] Therefore, we examine the dose dependence of glycoside hydrolases used to treat WT biofilm from 5% to 10% to 20%. We do not see any statistically-significant trends with increasing dosage of glycoside hydrolases (Figure S12 and S13). It may be that the biofilm compromise and dispersal previously seen with these hydrolases is not associated with significant changes in bulk biofilm mechanics. Our rheological studies are not sensitive to changes in adhesive forces (as long as the biofilm remains adhered to the rheometer tool, which it did in all cases), and the hour of treatment time may not be enough to fully capture the effects of enzymes on biofilms. If there are metabolic responses to the presence of enzymes, our assay is also unlikely to capture these changes—as we can see with there being no mechanical response to arginine treatment of biofilms, shown to be a disruptor via metabolic action by others.[43] In addition, some environmental conditions may enhance or diminish the action of these hydrolases; indeed, cellulase has been shown to be pH-sensitive, with greater biofilm inhibition at pH 5 than pH 7.[14]

We also do not find statistically-significant effects of DNase and alginate lyase on the mechanics of WT biofilms (Figure S14 and S15). It may be that these biofilms,

559 containing a mixture of Psl, Pel, and extracellular DNA (alginate is not an important
560 component of the PA01 matrix *in vitro* [50]), are not as vulnerable to targeted disruption by
561 a single specific enzyme as the PA01 variants examined earlier.
562
563 *Enzymatic treatment of biofilms grown in vivo*
564 It has previously been shown that biofilm dispersal in combination with antibiotic
565 therapy is an effective treatment for biofilm infections.[10-11] Dispersal happens when the
566 bacteria in a biofilm infection convert to the planktonic state; we anticipated that
567 compromising the biofilm matrix would promote dispersal by allowing bacteria to be more
568 easily released from the biofilm. Therefore, to assess the degree to which the *in vitro*
569 results described above may provide a guide to better methods of dispersing biofilm
570 infections *in vivo*, we tested the efficacy of alginate lyase and DNase I in inducing
571 dispersal of biofilms grown in a mouse model of chronic wound infection. Dispersal is
572 measured using the same procedure described earlier[11], as the percent of a sample that
573 is found in the planktonic state after treatment, given by the following fraction: (colony-
574 forming units in the fluid supernatant)/(colony-forming units in the supernatant plus
575 colony-forming units in the wound tissue).
576 We used bacterial strains that form Alg+, Psl+, and Pel+ biofilms *in vitro*; we have
577 not independently characterized the EPS composition of the corresponding biofilm
578 matrices for biofilms grown *in vivo*. For nominally-Psl+ biofilms, there was 48% greater
579 dispersal when treated with a mixture of alginate lyase and DNase than when treated with
580 a control (Figure 7); a Student two-tailed T-test gives a p-value of 0.003 for the null
581 hypothesis that the enzyme mixture and the control give the same dispersal from Psl+

biofilms, indicating statistical significance. For nominally-Alg+ biofilms, there was only a 16% greater dispersal when treated with the enzyme mixture than when treated with a control; for nominally-Pel+ biofilms, there was only a 3% greater dispersal when treated with the enzyme mixture than when treated with a control; these results are not statistically significant.

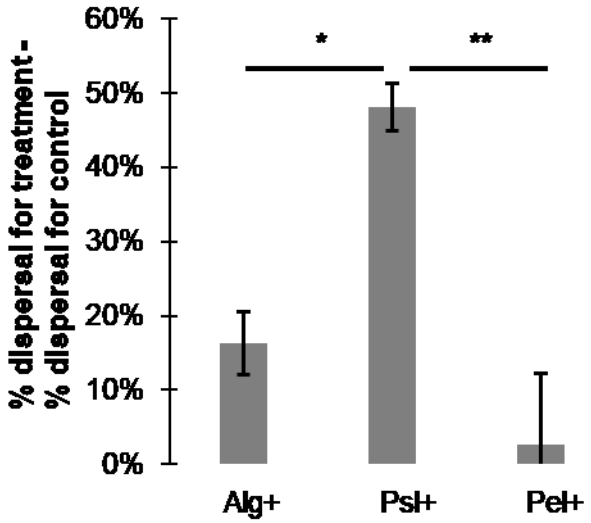

Figure 7. *Ex vivo*, a 500 μL of a mixture of 400 U/mL alginate lyase and 1000 U/mL DNase I elicits the greatest dispersal from Psl-dominant biofilms. Data shown are the dispersal measured for the treated sample minus dispersal measured for the enzyme-free control. When 3-day biofilms grown in the mouse chronic wound model are treated *ex vivo* with 200 U alginate lyase and 500 U DNase, Psl-dominant biofilms experience greater dispersal, compared with the control, than either alginate-dominant or Pel-dominant biofilms. * $p \leq 0.05$ and ** $p \leq 0.01$ from a single-factor ANOVA test and post-hoc testing using the Turkey-Kramer Multiple Comparisons test. Error bars are standard error of the mean. N=3 for each measurement.

Contrary to our expectation, these dispersal findings anti-parallel our mechanical results for biofilms grown *in vitro*, for which the greatest response was seen for Alg+ biofilms treated with alginate lyase and Pel+ biofilms treated with DNase I, with little to no effect of either enzyme on Psl+ biofilms. Furthermore, previous work has found that the generic glycoside hydrolases, which we find have only minimal effect on the mechanics of biofilms grown *in vitro* (Figure S10 – S13), result in 60% - 80% dispersal of *ex vivo* biofilms.[51] One explanation for this apparent discrepancy might be that the changes in biofilms associated with dispersal are not primarily mechanical in nature. Another possibility is that the matrix produced *in vivo* might not have the same population of polymers as that produced by the same strain *in vitro*, and therefore might not have appropriately specific targets for the alginate lyase and DNase I – except, perhaps, in the case of the nominally-Psl+ biofilm, which is the best-dispersed here despite being the least sensitive to alginate lyase and DNase I treatments when grown *in vitro*.

Conclusion

Summary of Results

For biofilms grown *in vitro*, alginate lyase and DNase I cleave alginate and eDNA polymers, respectively. For biofilms in which the target polymer is an important contributor to mechanics, this decreases yield strain and increases the diffusive transport of water through the biofilm matrix; we expect that this should correlate with increased diffusive transport of antibiotics as well. The increase in storage modulus that we find upon cleaving a dominant polymer type results from drying due to faster water transport,

621 and is therefore not a direct effect of cleaving the mechanically-dominant polymer; a
622 biofilm surrounded by a fluid or high-humidity environment, as it would be in the body,
623 would not dry and therefore would not experience an increase in storage modulus. The
624 dramatic effect of DNase I on Pel-dominant biofilms but not Psl-dominant or alginate-
625 dominant biofilms indicates that extracellular DNA is a dominant mechanical component
626 of the Pel polymer network, consistent with others' finding that Pel associates with eDNA
627 in the biofilm matrix.[25]

628 These results are not paralleled when we measure the effects of enzymatic
629 treatment on the dispersal of biofilms grown *in vivo*. This apparent discrepancy both
630 signals that dispersal may depend more strongly on non-mechanical properties than on
631 mechanical properties and highlights the importance of considering growth environment
632 when developing strategies for biofilm treatment. Growth in a living host could lead to
633 changes in polysaccharide production through changes in gene expression and to other
634 changes in matrix content through the incorporation of host material.[52-53] Understanding
635 how biofilm dispersal happens if biofilm mechanics is not an important contributor, and
636 how the content and enzymatic vulnerabilities of biofilm matrices depend on *in vivo* growth
637 conditions, both seem like worthwhile avenues of future research based on the findings
638 we present here.

639 Indeed, although *in vivo* animal models of infection (usually in mice) are widely
640 used to complement *in vitro* biofilm studies and to assess the degree to which *in vitro*
641 results may be extensible to *in vivo* scenarios, the current state of the art does not contain
642 *a priori* knowledge of how to map a set of *in vitro* biofilm properties to a transformed set
643 of *in vivo* biofilm properties. The *in vitro* and *in vivo* growth environments have many

644 differences in physics, chemistry, and biology, and it is not known which of these are
645 important for impacting biofilm characteristics. Establishing this knowledge would be a
646 large undertaking, but doing so would greatly increase the applicability of *in vitro* studies
647 such as the majority of the work in this paper.
648
649 *Future Directions: Other measurement methods*
650 Our rheological measurements primarily measure cohesive properties of the
651 matrix, being that the biofilms always remain fully adhered to the rheometer tool. Other
652 measurement methods should be used to complete the mechanical picture of these
653 polymer networks and reinforce rheological measurements. The mechanical contributions
654 of specific polysaccharide types to adhesive forces between the biofilm and a surface
655 have yet to be quantified, although it is known that Psl contributes to permanent biofilm
656 attachment, both Psl and Pel can play a role in initial attachment,[31, 54-57] and alginate is
657 not required for surface attachment.[58] In addition, how individual polysaccharides
658 contribute to properties such as wetting behavior and hydration of the biofilm network,
659 and how degradation of specific matrix polymers could give rise to alterations in the
660 diffusion of water, antibiotics, and nutrients is still understudied.
661
662 *Future directions: Biofilms of other species*
663 Many microbes other than *P. aeruginosa*, including many pathogens, also make
664 biofilms. Each biofilm-forming species makes different matrix polymers; indeed, even the
665 degree to which biofilm mechanics is dominated by matrix polymers (distinct from the
666 cells themselves) varies widely between organisms – *P. aeruginosa* is a copious producer

of matrix polymers but *S. aureus* and *S. epidermidis*, for example, are not, and their biofilm mechanics is largely influenced by direct cell-to-cell contacts. For both these reasons – different matrix polymer chemistries, and different amounts of matrix material in the biofilm – it is impossible to generalize our specific results on the effects of alginate lyase and DNase on Psl+, Pel+, and Alg+ *P. aeruginosa* biofilms to biofilms made by other species.. However, we do think it likely that some of the general principles revealed in this study – for example, that cleaving dominant matrix polymers, in a biofilm for which the matrix takes up a high volume fraction, increases diffusive transport within the biofilm and decreases the yield strain of the biofilm – are likely to be generalizable to many if not all species.

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Supporting Information

Figures S1 – S15

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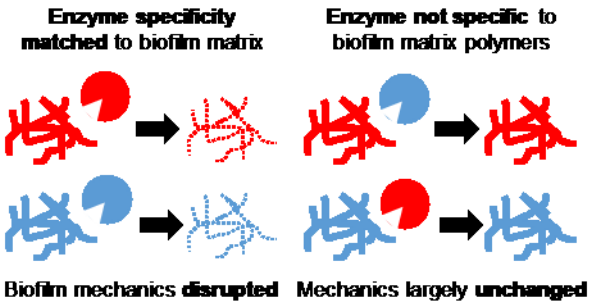

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