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**Article:**
Blakeman, J.T., Morales-García, A.L. orcid.org/0000-0002-4451-3225, Mukherjee, J. et al. (4 more authors) (2019) Extracellular DNA provides structural integrity to a Micrococcus luteus biofilm. Langmuir, 35 (19). pp. 6468-6475. ISSN 0743-7463

https://doi.org/10.1021/acs.langmuir.9b00297

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# Extracellular DNA provides structural integrity to a *Micrococcus luteus* biofilm

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**ABSTRACT:** Force spectroscopy was used to show that extracellular DNA (eDNA) has the pre-eminent structural role in a biofilm. The adhesive behavior of extracellular polymeric substances to poly(ethylene terephthalate), a model hydrophobic surface, was measured in response to their degradation by hydrolytic enzymes known for their biofilm-dispersion potential: DNaseI, protease, cellulase, and mannanase. Only treatment with DNaseI significantly decreased the adhesive force of the model bacterium *Micrococcus luteus* with the surface, and furthermore this treatment almost completely eliminated any components of the biofilm maintaining the adhesion, establishing a key structural role for eDNA.

## INTRODUCTION

Microbial life deposits on surfaces through polymer-encased assemblies known as biofilms. These sessile communities are considerably better protected than their planktonic counterparts. The extracellular polymeric substances that surround them provide resistance to biocides, antibiotics, desiccation, UV damage, shear forces, and predation. In some settings their dispersal is paramount, such as in biofilm-related infections (e.g. cystic fibrosis,[1] dental plaque,[2] and wounds[3]), medical implants and devices, and pathogen-ridden surfaces, where failure to remove them could cause morbidity and mortality.[4] In other areas, biofilm formation causes tremendous economic detriment, such as in the contamination of water supplies and water distribution systems,[5] textile staining and malodor in laundry,[6] biofouling of ship hulls,[7] and harboring food-spoilage microorganisms in preparation areas.[8] Thus, there is a need to develop biofilm dispersants that are able to rid surfaces of microbes and associated matrix polymers, ideally in a sustainable manner, that is, with low energy input and products of low toxicity and high biodegradability. Enzymes are ideal to fulfil this goal and consequently there is a huge industrial drive to harness their abilities. Having recognized the extent of biofilms on surfaces, the molecular mechanisms of their components must be understood.

Extracellular DNA (eDNA) is a pivotal component in biofilms. Being a large extracellular polymeric substance (EPS) and betraying large stiffness and charge,[9] it acts as both a bridge between bacteria and surfaces, to establish initial biofilms, and also between bacteria themselves, to enhance aggregation. Through a combination of acid-base, Lifshitz-van der Waals, and specific forces, eDNA forms a mesh in which it is bound to extracellular polysaccharides, forming dual fibrils,[10-12] and matrix proteins,[13-18] and is postulated to provide some support for the biofilm structure.[19] The sensitivity of eDNA to DNaseI enzymes which target it is critical to biofilm dispersal in a wider variety of Gram-positive,[10,11,20] Gram-negative,[21-23] and fungal organisms[24,25] than those for which cleavage of proteins and polysaccharides is significant. Although eDNA has synergistic interactions with other components in the EPS, treatment with only DNaseI is effective, particularly in young biofilms, suggesting that eDNA is a significant component in the integrity of the biofilm.[26] Hence, targeting eDNA could become a widely used approach against unwanted biofilms.

The removal of unwanted biofilms requires an ability to trigger dispersion of the bacteria on a macroscopic scale, which are already innate to biofilms. The life-cycle of biofilms rests on their flexibility and dynamic nature which allows them to respond to environmental cues, such as increased shear forces and starvation, by modifying their shape, mechanical properties, and niches.[27] This flexibility is given in part by the vast array of extracellular enzymes and surfactants that bacteria secrete and allows them to quickly adapt to the new conditions. A major translocation event would cleave critical *structural* macromolecules, whereas minor rearrangements would leave the primary structure intact and only modify the peripheral *adhesive* elements. Hence structural and adhesive components are both integral to the adaptability of biofilms. An understanding of the roles of macromolecules in the EPS is needed to target the key structural elements that would trigger massive dispersal events.

Here, the effect of different hydrolytic enzymes on the adhesion force of a live biofilm with a model hydrophobic surface has been determined using confocal microscopy

and force spectroscopy. Young biofilms of the Gram-positive organism *Micrococcus luteus* are intermittently attached to flat surfaces of poly(ethylene terephthalate) (PET), and their adhesive interactions recorded in turn in the presence of DNaseI, protease, mannanase, and cellulase. *M. luteus* is a non-pathogenic skin commensal, which is known to enhance the pathogenicity of *Staphylococcus aureus*[28] and consequently a relevant target in the study of medically-relevant polymicrobial biofilms.

*Micrococcus luteus* is commonly used as a model organism[29] by virtue of its sensitivity to enzymes,[30] ability to utilize a number of carbon sources,[31] ability to resuscitate from dormancy,[32] potential role in bioremediation,[33] and its known preferential attachment to hydrophobic surfaces such as PET.[6] An in-depth knowledge of how the EPS components are interwoven and of the molecular effect that targeting each component has on the adhesive profile of the biofilm, are needed to improve biofilm dispersal strategies[34] and for this reason force spectroscopy, a nanoscopic technique that elucidates the behavior of single molecules, was selected. Force spectroscopy has been little used to study the effect of enzymes on bacteria[35] and biofilms.[36] By analyzing the difference in adhesion events and the magnitude of the forces between *M. luteus* and PET in the presence of different enzymes, it is here shown that eDNA provides structural integrity to the biofilm of *M. luteus*, and that proteins have an apparent lesser structural role with polysaccharides having a secondary, non-structural function, being coadjuvants in the biofilm architecture.

## EXPERIMENTAL SECTION

Poly(ethylene terephthalate) (PET) pellets of number average molar mass $M_n$ = 27.5 kg/mol were used to create the thin films. Silicon wafers were obtained from Prolog Semicor, Ukraine. 2-Chloropentane (2CP) was obtained from Fluorochem (Hadfield, UK). 1,1,1,3,3,3-Hexafluoro-2-propanol (hexafluoroisopropanol, HFIP), glycerol, tryptic soy broth (TSB), tricine, sodium chloride, magnesium chloride, calcium chloride, tryptic soy agar (TSA), and poly-L-lysine hydrobromide with a molar mass of 30-70 kg/mol were purchased from Sigma-Aldrich (Gillingham, UK). LiveDead BacLight was purchased from Thermo Fisher Scientific. *Micrococcus luteus* ATCC 4698 was purchased from the American Type Culture Collection. Tipless silicon nitride cantilevers (NP-O10) were purchased from Bruker AFM Probes (Camarillo, CA, USA). Savinase 16L (protease), Carezyme Premium 4500L (cellulase), Mannaway 25L (mannanase), and DNaseI were obtained from Novozymes A/S (Bagsværd, Denmark). DNaseI (E.C. 3.1.21.1) is endogenous to *Aspergillus oryzae*, and its amino acid sequence is described in patent WO 2015/155350.

**Imaging buffer and enzyme preparations.** The imaging buffer consists of a tricine buffered solution of Milli-Q water at a pH of 8.4, supplemented with 200 mg/L of $Na^+$, 6.4 mg/L $Ca^{2+}$, 2.4 mg/L $Mg^{2+}$ and 250 mg/L $Cl^-$. These salt concentrations closely match those of the 10 % strength TSB, to minimize any environmental shock on bacteria caused by the imaging buffer upon removal from the growth medium. The enzymes (DNaseI, protease, mannanase, and cellulase) were tested at concentrations in imaging buffer ranging from 0.2 mg/L to 2 mg/L, chosen so that above this value the enzyme behavior was independent of concentration. This was close to 2 mg/L for DNaseI, but 0.2 mg/L for the other enzymes.

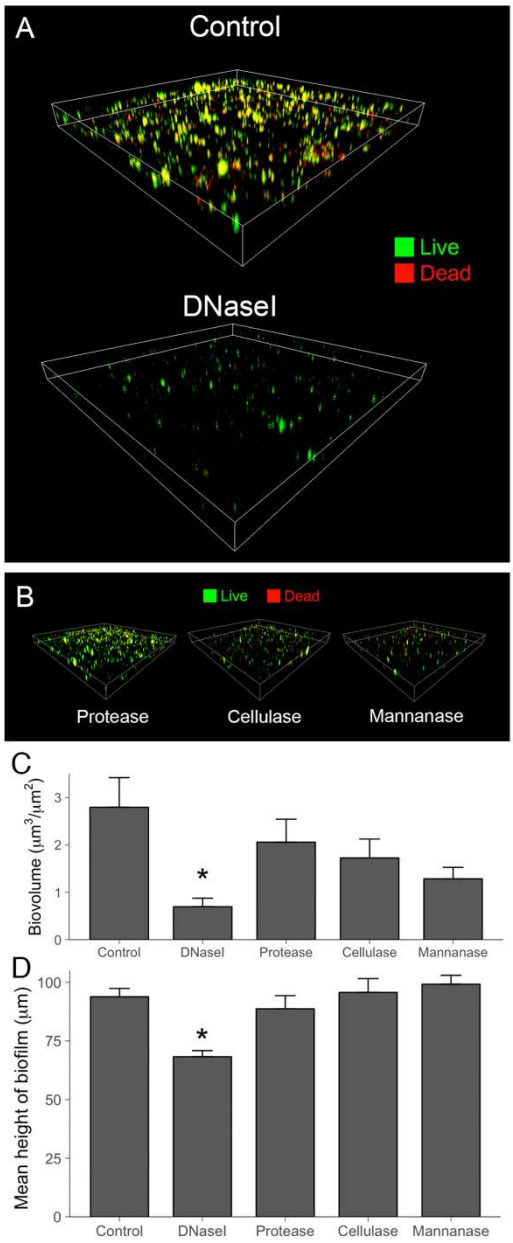


**Figure 1**. Impact of DNaseI, protease, mannanase and cellulase on 18 h biofilms of *M. luteus*. Biofilms were cultured on glass, stained with BacLight and visualized using confocal microscopy, taking *z*-stacks comprising seven stacks per image. Each image has lateral dimensions of 1.16 mm. Biovolumes were calculated from multiple confocal measurements taken across at least three slides for each enzyme. The chosen image for each enzyme is that with the biovolume closest to the mean result. (A) Control experiment (no enzyme) and the effect of DNaseI on the biofilm is shown. (B) The effect of protease, cellulase, and mannanase are shown. There was no significant ($p < 0.05$) decrease in biovolume from the control in any of these. (C) Bars represent mean average biovolume, with standard error shown by error bars. Asterisk indicates significant ($p < 0.05$) reduction in biovolume. (D) Mean height of all samples measured. The standard error is shown by error bars and the asterisk indicates significant ($p < 0.05$) decrease in biofilm height.

**PET Thin Film.** A 1% PET solution in 2:1 HFIP:2CP was spun onto silicon wafers at 2500 rpm for 60 s and annealed for 12 h at 200 °C under vacuum.

**AFM bioprobe construction.** *Micrococcus luteus* (ATCC 4698) were stored at –80 °C in 50% glycerol stocks and propagated onto TSA agar, incubating at 30 °C for 72 h. Liquid cultures were grown in TSB to stationary phase at 30 °C.

Tipless silicon nitride atomic force microscope (AFM) cantilevers were sterilized in 70% ethanol for 10 min and a polycationic layer attached onto them by adding 200 µL of 1% poly-L-lysine hydrobromide aqueous solution (w/w %) for 2 h. The cantilevers were then immersed in 500 µL *M. luteus* culture and incubated at 30 °C for 4 h. The cantilevers were then transferred to 1 mL of 10 % strength TSB and incubated at 30 °C for 18 h before the probe was used for adhesion measurements the next day.[37] Prior to each AFM experiment, the assembled probes were gently rinsed in imaging buffer (*vide infra*) to dislodge loosely bound bacteria.

**Confocal microscopy imaging.** Glass slides were prepared as the bioprobes were: using a 1% poly-L-lysine hydrobromide solution (w/w %) for 2 h before incubating in *M. luteus* culture for 4 h and then in dilute growth media for 18 h. Glass slides were submerged in imaging buffer containing the relevant enzyme (control slides used only imaging buffer) for 40 min before staining. To identify cells, slides were stained with BacLight (SYTO9 stain with excitation at 497 nm and emission at 543 nm and propidium iodide stain with excitation at 571 nm and emission at 638 nm) and rinsed gently before imaging in a Leica SP8 confocal microscope, employing images with a 10×/0.3 water dipped objective. Images were analyzed and biovolumes calculated using Imaris (Bitplane, Belfast, UK) software using the surface creation wizard with upper and lower intensity thresholds set to automatic. Data from the green and red channels were combined to give final values.

**Force measurements and enzyme treatment.** The forces of adhesion between the bioprobes and PET thin films were measured using an AFM operated in force spectroscopy mode (Asylum Research, MFP-3D), using a tipless cantilever with a nominal spring constant of 60 pN/nm. The true spring constant of each bioprobe was measured under the imaging buffer at room temperature, using thermal fluctuation[38] and all were found to be within the manufacturer's tolerances. Force acquisition was done using 10 × 10 force maps with a scan rate of 1 Hz, dwell time at the surface of 10 s, and retraction rate of 2 µm/s. The measurement had three experimental steps: (1) determination of the initial conditions (i.e. force acquisition under imaging buffer), (2) a 40-min incubation in enzyme-containing imaging buffer or fresh imaging buffer (control), and (3) measurement after the incubation period. Each experiment was conducted three or four times using a fresh bioprobe and hence in each case a unique biofilm was studied. Consequently, the 3-step protocol had to be conducted every time, to measure the relative changes in interaction force, since an absolute value cannot be measured and each bioprobe can only be compared to itself. The retraction curves were analyzed computing the adhesion force as the lowest value in the ordinate. The forces were statistically compared using a t-test with 95% confidence. The quantity, for force and extension of the secondary adhesion events were computed using a bespoke MATLAB algorithm.

**RESULTS**

**DNaseI disperses *M. luteus* biofilms.** Biofilms of *M. luteus* were cultured for 18 h on glass slides and their dispersal upon enzyme treatment was imaged using confocal microscopy (**Fig. 1**). After 40 min incubation, DNaseI was able to remove the majority of the biofilm with only tall spire-like structures remaining, suggesting that these were the oldest parts of the biofilm.[27] This supports other studies concluding that DNases are less effective against older biofilms,[26,39] which has been attributed to either eDNA being increasingly more shielded from the action of the enzyme by other biofilm components, or that eDNA is supplanted by other macromolecules. The other enzymes did not considerably decrease the biovolume of the biofilm after 40 min exposure.

The dispersal of the model biofilm was also studied using contact angle measurements (**Table S1**). The reduction in hydrophobicity of the biofilms after treatment was recorded, with DNaseI and protease lowering the angle of the assembly almost to the same level of the bare surface.

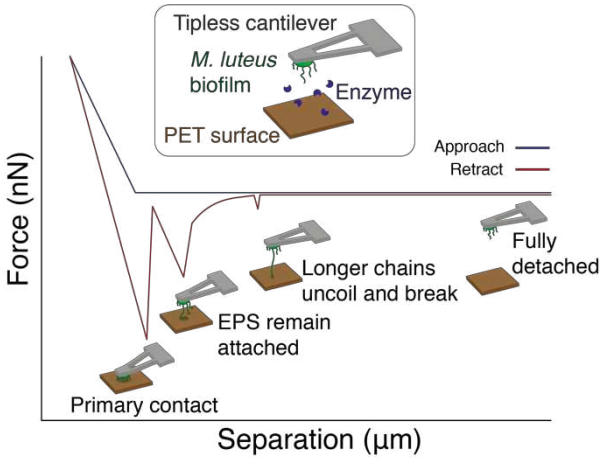


**Figure 2**. Schematic representation of a typical force-separation curve between a biofilm and a surface, showing the primary contact, from which the adhesion force is extracted, and minor peaks that correspond to the secondary adhesion events. (*Inset*) Experimental system used in these measurements.

**Structural changes through eDNA hydrolysis.** Fluorescence imaging combined with binding assays are widely used techniques that can give important information about the location of different components of a biofilm.[20,40,41] Such experiments have suggested roles for different components based on the morphology of the structures that they form. However, to assess a demonstrable role for these molecules, it is necessary to interact with the biofilm in a different manner. Here force spectroscopy was used to specify the effect that enzymes have on the interaction force between *Micrococcus luteus* biofilms and PET surfaces and thus to demonstrate the importance of eDNA to biofilm structure.

The bioprobes were first mounted on the piezoelectric mechanism of the instrument and repeatedly extended to contact the PET surface, whilst surrounded by enzyme-free imaging buffer. The contact time to ensure the consolidation of adhesive bonds between the bacteria and the surface in each cycle was 10 s. One hundred force-separation curves were acquired and their adhesion force (the ordinate of the primary contact in **Fig. 2**) was recorded. Once these initial conditions were measured, in the second step the imaging buffer was replaced by an enzyme-containing buffer, or enzyme-free buffer in the case of the control experiment. After 40 min incubation, on the third step, the biofilm was once again probed 100 times against the PET surface and the magnitude of the adhesion force compared to the initial conditions. The control experiments show a consistent drop of 32 ± 2% ($n = 3 \times 100$, $p \ll 0.05$), which is likely to be due to the mechanical stress placed on the biofilm during force measurements.[42] The enzyme experiments show a larger decrease in adhesion force of 92 ± 3% ($n = 3 \times 100$, $p \ll 0.05$) for the DNaseI, 52 ± 12% ($n = 3 \times 100$, $p < 0.05$) for the protease, 40 ± 5% ($n = 4 \times 100$, $p = 0.05$) for the mannanase and 53 ± 7% ($n = 3 \times 100$, $p < 0.05$) for the cellulase, indicating that *M. luteus* biofilms treated by hydrolases significantly decrease their adhesive force to a PET surface after a period of incubation. The biofilm was adsorbed on a poly-L-lysine-coated tipless AFM cantilever (**Fig. S1**), and no evidence of PET interaction with this polycation (**Fig. S2**) was observed. Consequently, the investigation of the effect of the enzymes was not prejudiced by the complete removal of the biofilm.

The reduction in adhesion after enzyme treatment suggests that either key binding components have been removed from the biofilm, reducing its interaction with the PET surface, or that the biofilm itself has been partly dispersed. Either possible mechanism indicates that the enzymes have cleaved the substrates that they target, namely phosphodiester bonds in eDNA (DNaseI), peptide bonds in extracellular proteins (protease), mannose-containing exopolysaccharides (mannanase), and β,1-4-glycosidic bonds in glucose-containing exopolysaccharides (cellulase) (**Fig. 3**), and that these components were used by the biofilm to attach to surfaces. The effect of using DNaseI is so large that it can be concluded that eDNA plays a crucial role in maintaining the adhesion of *M. luteus* to the PET surface.

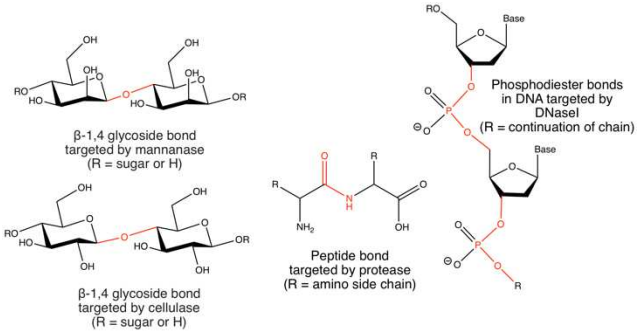


**Figure 3.** Examples of moieties in the EPS of bacteria targeted by the enzymes used in this work. The cleaved bonds are highlighted in red.

The force-separation curves (**Fig. 4**) obtained can also be analyzed in terms of the number of secondary events per curve (i.e. peaks of the retraction curve of less adhesive force than the primary contact, shown in **Fig. 2**). As the probe retracts from the surface, polymeric substances in the EPS that have become adhered to the substrate will be unwound and eventually rupture. These events are captured in the precise movement of the probe and recorded in the retraction curve. A bacterial biofilm can rapidly establish adhesive links when put in contact with a surface. If, upon enzymatic treatment, the number of secondary events per curve decreased, it would follow that a proportion of the links that maintained the biofilm-substrate interaction has been lost. As shown in **Fig. 5**, DNaseI has the greatest impact in the reduction of secondary events, followed by protease. The glycosyl hydrolases (mannanase and cellulase) cause little change in the number and distribution of events. Thus, the cleavage of eDNA in a *M. luteus* biofilm causes a dramatic reduction in the number of links that maintained its adhesion to a PET surface, more so than the hydrolysis of proteins and polysaccharides.

By analyzing the number of events after exposure to an enzyme (**Fig. 4**), the role of the targeted EPS component can be categorized as structural or adhesive. A structural component is here considered as a core element of the mesh that holds the biofilm together, so that its removal causes structural collapse and bacterial dispersion. This dispersion would be detected in force spectroscopy by a reduction in the number of anchor points (adhesion events) and a reduction in the force of adhesion. An adhesive component is here taken as a biomacromolecule that enhances the interaction capabilities of the periphery of the biofilm, which upon cleavage, diminishes its adhesive capacity, but does not critically affect the overall structure of the biofilm. The deterioration of adhesive components would then be characterized by a reduction in the strength of adhesion. Using these proposed definitions, it can be concluded that eDNA and proteins are both structural components, with the former having a larger effect in the overall structure, and that the polysaccharides functioning as non-structural, peripheral adhesive compounds.

**Extracellular DNA and proteins are structural components.** The force spectroscopy experiments (**Fig. 4**) suggested that eDNA and proteins are structural elements of the biofilm, as DNaseI and protease significantly reduced the number of adhesive contacts between a *M. luteus* biofilm and a surface (**Fig. 5**).

Extracellular DNA has been established as a functional component of the biofilms of many species and the ability of DNases to disperse biofilms has been recorded.[26,39] This group of enzymes randomly cleave phosphodiester bonds to form phosphooligonucleotide end-products. The force spectroscopy results presented here show that disrupting the backbone of eDNA causes a near total loss of adhesion (a 92 ± 3 % reduction in the force of adhesion). Given that upon DNaseI treatment there was a complete loss of secondary interactions in the majority of force-separation curves (80%), it can also be concluded that the degradation of eDNA also caused the disassembly of proteins and polysaccharides. Since it has been hypothesized that eDNA

is wound with polysaccharides forming fibrils or anchored to proteins forming a mesh,[16,17] it is possible that the loss of eDNA, the key cohesive element of the mesh, dispersed the assembly.

Proteins are also key elements of biofilms, performing structural and protective roles.[43] Proteases hydrolyze peptide bonds in proteins and in these experiments the cleavage of this component provoked a decrease of 54 ± 12 % in the adhesion of the biofilm to PET. Although substantial, the fact that some adhesion remains indicates that either eDNA and polysaccharides on their own can support the adhesion to the PET substrate or that an assembly of these and other biofilm matrix components render a number of proteins inaccessible.

**Polysaccharides are adhesive components**. The force spectroscopy experiments (**Fig. 4**) suggested that β-1,4-linked glucans and mannans are adhesive elements of the biofilm, as cellulase and mannanase reduced the adhesion between a *M. luteus* biofilm and a surface, whilst leaving the number of bonds virtually unchanged (**Fig. 5**)

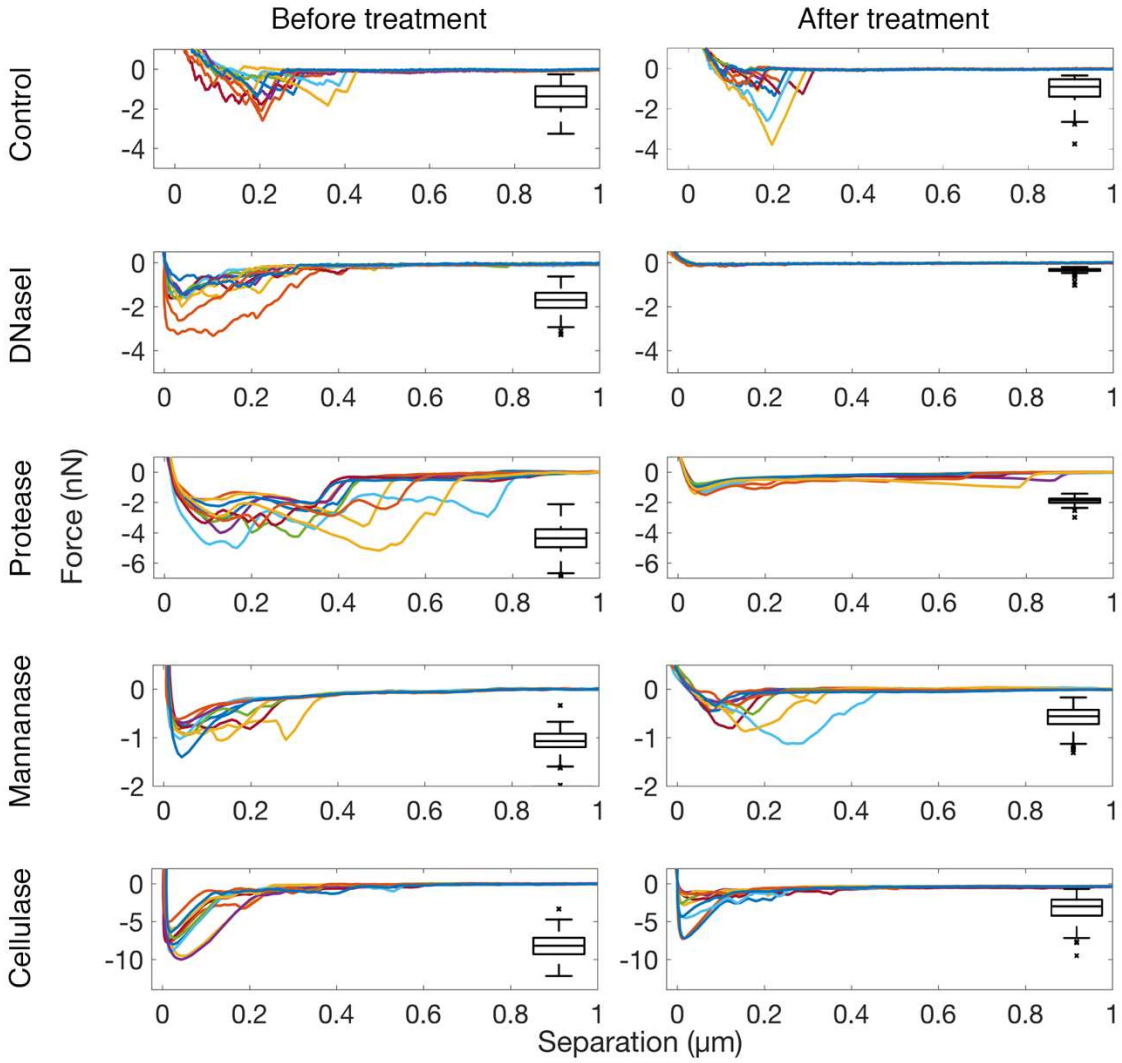


**Figure 4.** Force-separation retraction curves before (left) and after (right) treatment with specific enzymes. Each plot overlays 10 *randomly* selected curves and is representative of all measurements. The boxplots denote the distribution of adhesion, i.e. the magnitude of the primary peak of adhesion. Each boxplot corresponds to a single pair of force maps, comprising 100 data each. The top and bottom edge of the box are the 25th and 75th percentiles (quartiles) of the data, with the middle bar being the median value. The whiskers extend from their respective quartile to a spread of 1.5 times the interquartile range. Any data outside of this range is marked on the plot by a cross. The data marked "before treatment" were obtained using the same biofilm as the corresponding data marked "after treatment". The ensemble of "before treatment" panels are rather different, however and this indicates the level of reproducibility in the experiment.

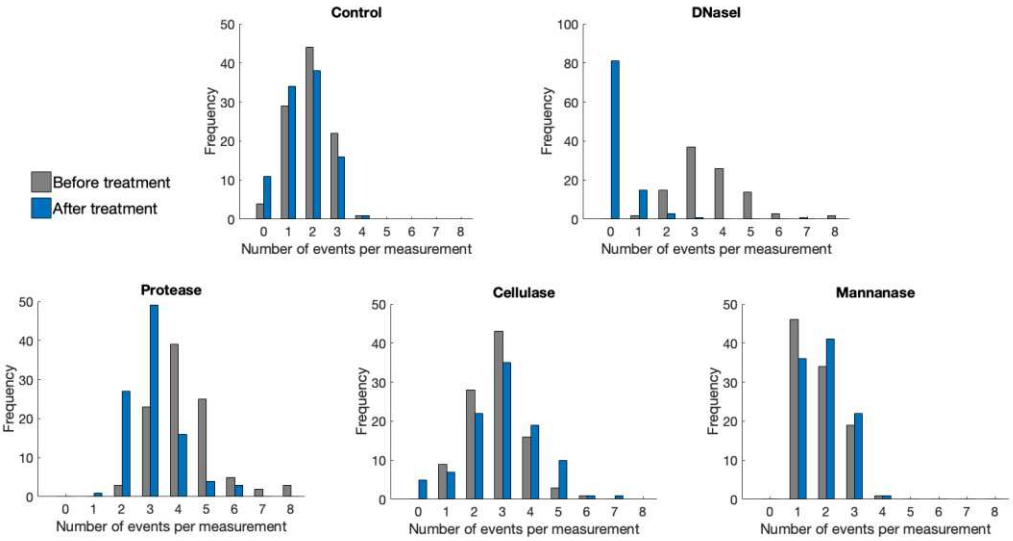


**Figure 5**. Bar charts showing the number of secondary events per force-separation measurement before and after treatment with each enzyme. Each plot corresponds to a single pair of force maps, comprising 100 data each.

Polysaccharides are an integral part of the biofilm and can be composed of a wide variety of sugar monomers, linked with different connectivity and stereochemistry. Common polysaccharides in biofilms include β-1,4-D-glucans and β-1,4-D-mannans, and although research on the polysaccharide composition of *M. luteus* has not been extensive, the presence of lipomannans has been reported,[44] forecasting their susceptibility to mannanases. In other assays, cellulases and mannanases have been used to disperse lab strains,[45] medical,[46,47] and industrial[48] biofilms and these enzymes are commonly used in biofilm-dispersing compositions.

The mannanase used in this work is a glycosyl hydrolase belonging to the GH5 group, specifically to the enzyme class E.C.3.2.1.78 that catalyzes the endohydrolysis of (1→4)-β-D-mannosidic bonds. The use of mannanase in *M. luteus* decreased its strength of adhesion to PET by 40 ± 5 %, having the smallest effect of all the enzymes tested. Upon treatment, the distribution of the number of secondary events per curve remained constant and thus no structural changes have been ascribed to the mannan component of the biofilm.

The cellulase used here is a glycosyl hydrolase that belongs to the GH45 or E.C.3.2.1.4 class and as such it catalyzes the endohydrolysis of (1→4)-β-D-glycosidic bonds. Cellulase reduced the biofilm-substrate adhesion substantially (53 ± 7 %). This large decrease in the strength of adhesion indicates that polysaccharides containing these linkages are important for the interaction of *M. luteus* biofilms to a hydrophobic substrate. The analysis of the secondary events reveals that the number of attachment points remain unchanged and hence the role of cellulose-like polymers in this biofilm has been categorized as adhesive.

## DISCUSSION

Biofilm architecture can vary substantially, since biofilms are complex, heterogeneous and dynamic consortia. Notwithstanding this variability and given that eDNA is ubiquitous in the environment, eDNA is used as a functional element in biofilms of Gram-positive and Gram-negative bacteria, as well as in fungi. The success of the eDNA biofilm element across kingdoms and phyla is due to its ability to interact with other extracellular substances to form components that join the internal structure of the biofilm. Dual fibrils of eDNA and polysaccharides have been observed in the Gram-positive *Streptococcus mutans*,[11] *Enterococcus faecalis*,[49] *Staphylococcus epidermidis*,[20] and *Listeria monocytogenes*[10] and the Gram-negative Enterobacteriaceae,[50] *Myxococcus xanthus*,[36] and *Pseudomonas aeruginosa*.[41] On the other hand, proteins and eDNA form a mesh, in which proteins are critically positioned at junctions, binding eDNA in a lattice form.[18] This dual contribution to the biofilm scaffold has been observed in the Gram-positive *S. aureus*, *S. epidermidis*,[15] *Streptococcus pneumoniae, S. mutans*[14] and in the Gram-negative *Haemophilus influenzae*,[39] *Escherichia coli*,[51] *Moraxella catarrhalis*,[14] *Neisseria meningitidis*,[13] and *P. aeruginosa*.[12] Thus, the knowledge of the enhancement that eDNA brings to the assembly has opened new possibilities for biofilm control.[14,16,18,39] By strategically targeting eDNA, and by knowing the role that other EPS components have in shielding this nucleotide, improved strategies for addressing the deleterious effects of biofilms can be developed.

Cleavage of eDNA does not cause a large change in thickness in the biofilm (a decrease of 27.2%, **Fig. 1D, Table S3**) but does cause the loss of most of the cells (**Fig. 1C**). The combination of these points suggests that the biofilm has become porous or has some other decrease in density (the data in **Fig. 1D** do not preclude significant loss of material), possibly due to the removal of a contiguous eDNA-rich structure.

Force spectroscopy is able to give detailed information about how the biofilm binds to a preferential surface. The primary event (**Fig. 2**) usually has the largest adhesive force and indicates how strongly the biofilm and substrate are attached to each other. The secondary peaks give finer details about the nature of the binding. After enzyme

treatment, if the primary event decreases in magnitude, but a similar number of secondary peaks remain, some bacterial dispersion has taken place, without much change in the density of adhesive contacts. The interactions remain the same, there is just less adhesive material present. The substance targeted by the enzyme is therefore classified as adhesive in nature. This is supported by contact angle measurements (**Table S1**), which show that the glycosyl hydrolases (cellulase and mannanase) cause minimal change to the surface energy of a biofilm on a silicon wafer.

If the secondary events do alter, a change in the structure of the biofilm can be concluded. Whole sections of the biofilm may have been cleaved and jettisoned, taking adhesive material with it. eDNA and proteins are categorized as structural components for this reason.

With a change in secondary events, we can conclude there has been a change in the structure of the biofilm. When eDNA is targeted, polysaccharides and other components are lost, leading to the classification of eDNA as a structural component. Because of the large loss of material after DNaseI treatment, it is not possible to ascertain the level of contribution that eDNA has to the adhesive properties of the biofilm.

Targeting proteins also changes the distribution of secondary events, albeit to a lesser extent than the targeting of eDNA. However, the change in contact angle after protease treatment (**Table S1**) suggests there is a minor change in the biofilm structure that allows greater ingress of water. Therefore, protease is categorized as causing a minor structural change in the biofilm.

**Conclusion.** In the present work, the study of the effect of enzymes in the dispersal of the model biofilm-forming species *Micrococcus luteus* and its adhesiveness to a model hydrophobic poly(ethylene terephthalate) surface, were studied. The adhesiveness of the biofilm was measured using force spectroscopy, where force-separation curves were obtained prior to and following enzymatic treatment with a DNaseI, protease, mannanase, and cellulase. The hydrolysis of phosphodiester bonds by DNaseI eliminated virtually all adhesive contacts between the biofilm and the PET surface, decreasing the interaction force dramatically. Similarly, targeting peptide bonds of extracellular proteins using a protease led to a decrease in the force of adhesion and the number of cell-surface interaction points.

Because their cleavage provokes a massive dispersal event and the reorganization of the biofilm, a *structural* role is ascribed to eDNA and extracellular proteins. Conversely, targeting glycosidic and mannosidic bonds in exopolysaccharides led to a decrease in adhesion but left the number of anchor points largely unchanged, and thus an *adhesive* role was attributed to these polysaccharides.

The use of confocal microscopy in tandem with force spectroscopy leads to logical answers to the changes being observed in these complex arrangements of bacteria and polymeric substances. This work demonstrates the importance of perturbing the biofilm (here by force spectroscopy) to obtain key adhesive and structural information, rather than relying simply on optical images.

This has been a comprehensive study of the fate of the extracellular molecules of a biofilm after enzymatic treatment and this information aids in the construction of a model in which the role of molecules can be understood. By using force spectroscopy to study the interaction between a biofilm and a surface, the behavior of extracellular polymers experiencing chemical inputs can be seen in real time, making it a valuable tool in the measurement of enzyme efficacy, which traditionally has been done using macroscopic methods. By identifying efficacious enzymes and understanding their discrete effects in the behavior of the whole assembly, new targets for biofilms can be identified and superior enzymes can be designed to concoct dispersants with medical and industrial applications. Most importantly, this work can help in the understanding of biofilm eradication of other species and polymicrobial consortiums in which DNases show superior dispersion. Ultimately, approaches can be designed for the degradation of the proteins that anchor the eDNA and the polysaccharides that strengthen the intercellular adhesion.

## ASSOCIATED CONTENT

**Supporting Information**. Contact angle methods, results of biofilm coated silicon wafer after enzyme treatments, fluorescence image of biofilm-coated cantilever, and force-distance control curves showing poly-L-lysine contacting a PET substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Conflict of Interest Disclosure

Procter and Gamble and Novozymes declare commercial interests in the use of DNaseI for cleaning applications.

## ACKNOWLEDGMENT


JTB thanks the EPSRC for an iCASE studentship, aligned with the EPSRC CDT in Polymers, Soft Matter, and Colloids. Drs. Matt Mears, Robert Turner, and Andrew Parnell are respectively thanked for their help with contact angle measurements, bacterial growth, and the preparation of the PET films. Mr. Michael L. Groombridge is thanked for his assistance with the analysis of confocal images.


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