This postprint is published in:

***Soft Matter*, 2019, 15, 5042-5051, DOI: 10.1039/C9SM00297A**

Viscoelastic response of *Escherichia coli* biofilms to genetically altered expression of extracellular matrix components

Maruša Horvat¹, Archana Pannuri², Tony Romeo², Iztok Dogsa¹, David Stopar^{1}*

¹*University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Večna pot 111, 1000 Ljubljana, Slovenia*

²*University of Florida, IFAS, Department of Microbiology and Cell Science, P.O. Box 110700, Gainesville, FL 32611-0700, USA*

*Correspondence to:

Dr. David Stopar

Chair of Microbiology, Department of Food Science and Technology

Biotechnical Faculty, University of Ljubljana

Vecna pot 111

1000 Ljubljana, Slovenia

Tel: +386 1 320 3412

Fax: +386 1 257 33 90

Email: david.stopar@bf.uni-lj.si

Key words: biofilm, *E. coli*, EPS, rheology, viscoelasticity

Abstract

How viscoelastic properties of the extracellular matrix affect the various biological functions conferred by biofilms is an important question in microbiology. In this study viscoelastic response of *Escherichia coli* biofilms to genetically altered expression of extracellular matrix components was studied. Biofilms of the wild type *E. coli* MG1655 and its mutant strains producing different amounts of extracellular matrix components (curli, colonic acid, poly- β -1,6-*N*-acetyl-D-glucosamine) were used to examine the viscoelastic behavior of biofilms grown at solid - atmosphere interface. The results suggest that the presence of curli proteins dominates biofilm mechanical behavior. The rheological data indicate that cohesive energy of the biofilm was highest in the wild type strain. The results demonstrate the importance of extracellular matrix composition for biofilm mechanical properties. We propose that by genetically altering the expression of extracellular matrix polymers bacteria are able to modulate the mechanical properties of their local environment in accordance with bulk environmental conditions.

Introduction

Biofilms are viscoelastic structures of bacterial cells and extracellular polymeric substances (EPS), which enable cell-cell interactions and adhesion to surfaces.¹⁻⁴ EPS is a multi-component substance,⁵⁻⁷ composed of polysaccharides, proteins, and eDNA that reacts in a time-dependent manner to externally induced mechanical stress with a combination of elastic and viscous responses. The resulting viscoelasticity is a contributing factor to the resistance of biofilms to both mechanical and chemical challenges.⁸ Whereas, elasticity is usually the result of molecular stretching, viscosity is a direct result of a molecular flow.⁹ It is expected that the molecular composition of the EPS will have a significant impact on biofilm viscoelastic properties. Currently it is not known how different EPS components affect the mechanical properties of *E. coli* biofilms.

Several microscopic techniques allow the imaging of high resolution biofilm structures such as transmission electron microscopy (TEM),¹⁰ scanning electron microscopy (SEM),¹¹ environmental SEM,¹² and focused ion beam scanning electron microscopy (FIB-SEM).¹³ Due to requirements for some degree of sample preservation, conventional electron microscopy techniques are often

susceptible to sample distortion and artifacts; for example, the EPS may appear as fibers rather than as a thick gelatinous matrix surrounding the cells.¹⁴ Although a wet or partially hydrated state has been used to observe biofilm with environmental SEM or frozen-hydrated biofilms with FIB-SEM, these techniques are unable to reveal the dynamic architecture of internal 3D structures inside the highly hydrated EPS.^{12,15} The internal 3D biofilm structure can, however, be determined by confocal laser scanning microscopy (CLSM), which enables structure determination in hydrated state, but requires fluorescence labeling of structural components. A limiting factor in the use of CLSM is that penetration of the fluorophores and laser light into biofilm is typically confined to 20 to 40 μm ,¹⁶ and it is difficult to obtain images from deeper within a biofilm, which can often exceed 100 μm in thickness. In addition, 3D biofilm structural responses to applied fluid shear stresses have been measured by optical coherence tomography.²⁰ Although microscopic techniques have yielded a general description of biofilm structure and to a limited degree its microbiological and biochemical composition, there are certain aspects of biofilm structure that are not suitable for analysis by microscopy. In particular, viscoelastic dynamic properties of biofilm material. Viscoelastic properties of a biofilm reflect its internal structure and determine its response to the externally induced mechanical stress, which is important in biofilm proliferation and eradication. The bulk viscoelastic properties of biofilms can be measured by rotational rheometry.^{3,17} By performing microbead force spectroscopy with a closed-loop atomic force microscope, relaxation processes, and nanoindentation it has been possible to monitor rheology of biofilms.¹⁸ One of the distinct features of AFM is its ability to resolve mechanical properties of materials at the nanoscale. In contrast to rheological tests, AFM focuses on a very small area, which does not allow one to determine the response of the entire structure to the mechanical stresses, but only from individual molecules. A general conclusion from these studies is that there exists a correlation between quantifiable viscoelastic properties of a biofilm and its structure and composition.

Upon mechanical deformation, biofilm structure rearranges. Bacterial cells, which are typically the most abundant and massive particles in the biofilm, respond slowly to deformation. For example, *P. aeruginosa* cells in a biofilm could not reach new stable positions within 100 s upon an imposed deformation, as determined by direct microscopic observation of relaxation processes.²¹ On the other hand, EPS components relaxed within 20 s. This suggest that biofilm

network remodeling primarily involves changes in extensive extracellular polymer contacts, which can reestablish quicker than contacts between bacteria or between bacteria and EPS. In general, two principal components suffice to describe stress relaxation of biofilms.²² The fastest principal relaxation component is associated with the flow of water and soluble solutes into the porous system of biofilm. A second principal relaxation component is associated with the EPS matrix.

It has been demonstrated that ratios of the different EPS components can vary depending on the environmental conditions,²³ as well as genetics of the strain.²⁴⁻²⁶ In this work, we systematically alter gene expression of extracellular matrix components to study mechanical response of the *E. coli* MG1655 biofilm superstructure. *E. coli* is a diverse collection of strains that includes inhabitants of the commensal flora of the human and animal intestinal and urinary tracts. In these environments, bacteria are regularly exposed to shear stress.⁵⁶ The relationship between EPS and biofilm mechanical properties of *E. coli* is largely unknown. In contrast, the composition and regulation of EPS matrix component biosynthesis are well described. Four major EPS components have been identified in different *E. coli* biofilms: PGA, a linear polymer of β -1,6-*N*-acetyl-D-glucosamine containing some deacetylated glucosamine residues;²⁷⁻²⁹ curli proteins;³⁰ colanic acid, a branched polymer containing glucose, fucose, galactose and glucuronate;^{31,32} and bacterial cellulose or cellulose derivatives.^{33,34}

The EPS of the wild type *E. coli* MG1655 biofilm examined in this study contains modest amounts of PGA and curli.^{29,35} This strain does not produce cellulose.³⁶ The disruption of the *csrA* gene in MG1655, which encodes an RNA binding protein and a global regulator,³⁷ leads to a dramatic increase in biofilm formation and PGA biosynthesis via multiple regulatory circuits.³⁸⁻³⁹ PGA production is increased under high Na⁺ levels (e.g. LB), low temperature and is high in the *csrA* mutant. To increase curli production, deletion of the *pgaC* gene and disruption of the *cpsE* gene by transposon has been proposed. Expression of curli is increased under low osmolarity and low temperature conditions. The production of colanic acid under normal growth conditions in MG1655 is limited by production of the regulatory protein RcsA. Under normal growth conditions RcsA is poorly translated and is degraded by Lon protease,⁴⁰ which in turn limits colanic acid synthesis. Mutant strains of *E. coli* MG1655 used in this study expressed different combinations of

extracellular matrix components. The results suggest that alteration of extracellular matrix composition can significantly affect the mechanical properties of the resulting biofilm.

Materials and methods

Bacterial strains and growth conditions. The properties of bacterial strains used in this study are presented in Table 1. For rheology experiments bacteria were grown in liquid LB medium and on 2 % LB agar plates. For bacterial suspension experiments, bacteria were cultivated overnight in 5 mL of LB medium amended with appropriate antibiotic (10 µg/ml of tetracycline for *pgaC* mutant and 100 µg/ml kanamycin for other mutants) at 37 °C.³⁵ To grow biofilms, the 0.1 ml of overnight culture (37 °C, 250 rpm) in liquid LB medium with corresponding antibiotics was spread onto 2 % LB agar plates with corresponding antibiotics, and incubated overnight at 37 °C. For growing biofilms on microscopy slides 50 µL of overnight culture was incubated in 25 mL of liquid LB medium supplemented with corresponding antibiotics in 50 ml centrifuge tubes with slides positioned in a vertical direction for 24 hours at 37 °C without agitation. After 24 hours of incubation slides were transferred to new tubes with fresh growth medium and incubated for additional 24 hours at 37 °C without agitation.

Table 1: Bacterial strains used in this study.

Strain	Name	Genotype	Phenotype	Extracellular polysaccharides produced by the strain	The source of strain
<i>Escherichia coli</i> MG1655	<i>wt</i>	<i>wt</i>		PGA, curli	<i>E. coli</i> Genetic Stock Center (CGSC#6300)
<i>Escherichia coli</i> MG1655	<i>pga</i>	$\Delta csgA$ <i>cpsE::Tn10</i> <i>csrA::kan</i>	Deletion of <i>csgA</i> gene, <i>cpsE</i> gene interrupted by tetracycline resistance gene, <i>csrA</i> gene interrupted by kanamycin resistance gene.	PGA (overproduction)	This study.
<i>Escherichia coli</i> MG1655	<i>crl</i>	$\Delta pgaC$ <i>cpsE::Tn10</i>	Deletion of <i>pgaC</i> gene, <i>cpsE</i> gene interrupted by tetracycline resistance gene.	curli	This study.
<i>Escherichia coli</i> MG1655	<i>cla</i>	$\Delta csgA \Delta pgaC$ $\Delta lon::kan$	Deletion of <i>csgA</i> , <i>pgaC</i> and <i>lon</i> genes, insertion of kanamycin resistance gene.	colanic acid (overproduction)	This study.
<i>Escherichia coli</i> MG1655	<i>eps-</i>	$\Delta csgA \Delta pgaC$ $\Delta lon::kan$ <i>cpsE::Tn10</i>	Deletion of <i>csgA</i> , <i>pgaC</i> and <i>lon</i> genes, insertion of kanamycin resistance gene, <i>cpsE</i> gene interrupted by tetracycline resistance gene.	does not produce PGA, curli, colanic acid	This study.
<i>Bacillus subtilis</i> PS216		<i>wt</i>			University of Ljubljana, Biotechnical Faculty ⁴¹

Biofilm Formation Assay. Strains of *E. coli* were assayed for biofilm formation (crystal violet staining) in LB medium in 16 x 100 mm glass tubes or in MOPS minimal medium with 0.4% Glucose (<https://www.genome.wisc.edu/resources/protocols/mopsminimal.htm>) in 96 well polystyrene microtiter plates, as previously described.⁵⁵

Congo red binding assay. Five microliters of overnight cultures of different *E. coli* strains were inoculated onto LB agar plates incorporated with Congo red (40 µg/ml) and incubated at 28 °C for 48 h, before being photographed.

Rheology of biofilms.

Rheological measurements were performed on a rotational rheometer Physica MCR 302 (Anton Paar, Graz, Austria) at (20.00 ± 0.01) °C. After growing biofilms on agar plates for 48 hours the biofilms were extracted from the agar plates by carefully scratching the surface with glass slides. The sample was placed in the center of the lower rheometer plate using a pipette tip. Both PP25 and CP25-1 measuring systems were used for biofilm measurements with approximately 0.07 ml of biofilm. This low volume of sample was used because it was difficult to obtain enough biofilm material for replicate measurements in larger quantities. The gap between the upper and lower measuring plate was reduced to the trim position. The trim gap position was 10 μm higher than the measuring gap position. We routinely determined that the gap was completely filled with the sample. With a trimming tool we removed any excess material along the brim of the plate, so that the sample corresponded to the external diameter of the upper plate. Next, the final measuring gap of 48 or 100 μm was approached for CP25-1 and PP25 respectively. During the compression phase of the sample the normal force was constantly monitored on the rheometer and did not exceed 2N. For bacterial suspensions the sample volume was not constrained, and the CP50-1 system was used with approximately 0.6 mL of bacterial suspension applied to the measuring system.

Viscosity was measured at shear rates ranging from 0.1 to 1000 s^{-1} in 25 logarithmically spaced steps.^{19,42} It has been reported previously that in the gastrointestinal track, a common environment of *E. coli*, during vomiting, bowel movements, and defecation the maximal shear rates can reach 1000 s^{-1} or higher.⁵⁶ Oscillatory amplitude sweep test was performed at the angular frequency ω of 10 s^{-1} and the strain γ ranging from 0.01 to 100 % in 20 logarithmically spaced steps. Cohesive energy (CE) was calculated from the amplitude sweep to determine stability of the biofilm as $\text{CE} = \frac{1}{2} G' \gamma_c^2$, where G' is storage modulus value in the linear viscoelastic region and γ_c^2 is a critical strain at the end of the linear viscoelastic region. The cohesive energy density is usually expressed as cohesive energy per unit volume and has a unit of J/m^3 . The larger the cohesive energy per unit volume, the more stable is the biofilm. The yield point is a shear deformation at the upper limit of linear viscoelastic region, when G' starts to decrease. The yield strain point was determined as a shear strain where the G' value deviated from the linear viscoelastic region line by more than 10

%. The flow point was determined as a shear strain at the crossing of the G' and G'' curves, i.e. when $G' = G''$. The flow transition index was calculated as a ratio of flow point to yield point. All rheological experiments were done at least in triplicate. To test the effect of moisture, biofilms were grown on agar plates and were covered with water or 41 mM SDS solution and allowed to absorb the liquid for one minute. Next, by tilting the plate the excess liquid was removed from the biofilm and viscosity of the hydrated biofilm was measured.

Antibacterial treatment. A viability test with BacLight Bacterial Viability Kit (SYTO 9/propidium iodide, Invitrogen, 2009) was carried out to determine how effectively hydrogen peroxide killed cells from different biofilms. Bacterial biofilms were grown on microscope slides as described above for 48 hours. Then, the biofilm microscope slides were removed from the media and inserted into centrifuge tubes containing either 6 % hydrogen peroxide or distilled water (for control) and incubated for 20 min at room temperature. Next, the slides were rinsed with phosphate buffer solution (PBS) and stained with BacLight Bacterial Viability Kit followed by 30 minutes of incubation at room temperature. To each slide 20 μ L of stain mixture (1 mM SYTO and 6 mM propidium iodide in PBS) was added and covered with a glass cover slip (24 $\times$ 60 mm). Cells with an intact cell membrane emit only green light due to SYTO 9 fluorescence (Em. 480/Ex. 500). On the other hand, cells with a compromised cell membrane (dead cells) emit red light due to the interaction of propidium iodide with nucleic acids (Em. 536/Ex. 617). The samples were observed with epifluorescence microscope Axio Observer Z1 (Zeiss, Göttingen, Germany) with appropriate settings for fluorescence (filter sets 38HE and 43HE). The differential interference contrast (DIC) and brightfield images were observed using 10 $\times$ /0.3 and 20 $\times$ /0.4, Zeiss objectives. Images were recorded with a coupled MRm Axiocam camera (Zeiss, Göttingen, Germany).

To calculate the fraction of dead cells, at least six randomly selected view fields per individual biofilm zone were examined and analyzed with ImageJ to determine the number of live and dead cells. The total number of cells were obtained from DIC images, which proved to be a more reliable proxy for a total bacteria count compared to a sum of green and red fluorescent cells. The number of dead cells were obtained from red fluorescent cells. In total, from 1000 to 30,000 cells

were examined. The fraction of the dead cells in a given field was calculated by dividing the number of red fluorescent cells by the total number of cells.

Results

In this study, rheological characteristics of a wild type (wt) *E. coli* MG1655 biofilms and four of its mutant strains with mutations affecting the production of various extracellular polymers were compared. We examined the effects of extracellular polymeric substances on biofilm formation in LB medium (Fig. 1 panel A) and in MOPS minimal medium with 0.4 % Glucose (Fig. 1 Panels B and C). The *pga* strain ($\Delta csgA$ *cpsE::Tn10* *csrA::kan*) showed strong crystal violet staining (Panel A), indicative of copious biofilm formation, consistent with the effect of the *csrA* mutation on PGA-dependent biofilm formation in LB medium.⁵⁷ The *crl*, *cla* and *eps*- strains showed crystal violet staining similar to the wt strain in LB medium. In contrast, wt *E. coli* showed the greatest crystal violet staining in MOPS minimal medium with 0.4 % glucose (Fig. 1 panel B and C). We also tested colony morphology and binding of the diazo dye, Congo red, to visualize differences in curli and PGA production by these strains.⁵⁸⁻⁵⁹ Colonies of wt, *pga* and *crl* strains were red in color (Panel D) indicative of binding of Congo red to both PGA and *crl*, while the *cla* and *eps*- strains did not bind the dye.

Figure 1: Biofilm assay and Congo red binding of *Escherichia coli* strains. *E. coli* strains used in the study and an uninoculated control were assayed for biofilm formation in LB in 16 x 100 mm test tubes and photographed after staining with crystal violet (Panel A). Growth (A_{600}) and crystal violet staining of biofilm formation (A_{630}) by cultures grown in liquid MOPS minimal medium with 0.4% glucose in a 96 well polystyrene plate (Panel B). The original wells of the plate prior to solubilization of crystal violet (panel C). Five microliters of overnight cultures of *E. coli* strains were inoculated onto LB agar containing Congo red (40 $\mu\text{g/ml}$) and incubated at 28 $^{\circ}\text{C}$ for 48 h (Panel D).

To compare the viscosity of bacterial suspensions and biofilms we measured viscosity curves (Fig. 2). The results indicate several orders of magnitude higher viscosity of biofilms compared to bacterial liquid suspensions. The shape of the viscosity curve of bacterial suspension revealed only a small pseudoplasticity (decreasing viscosity with increased shear rate). At high shear rates the viscosity of a bacterial suspension was similar to that of water. The viscosity curve of a biofilm, on the other hand, showed a strong pseudoplastic behavior. The viscosity dropped over two orders of magnitude in the shear rate range from 1 to 1000 s^{-1} . Nevertheless, at high shear rates the biofilm still exhibited a high viscosity indicating that biofilm internal structure was not completely

disrupted. Because the network structure was much more pronounced in biofilms compared to liquid cultures, we focused our subsequent experiments on biofilms only.

Figure 2: Viscosity curve of biofilm (black) grown on 2 % agar for 48 h, bacterial suspension (red), and distilled water (blue). The average values and standard errors of the mean are given ($n = 5$ for bacterial suspension, $n = 11$ for biofilm).

The viscosity of biofilms grown on agar plates for 24 h was dependent on agar concentration (Fig. 3). Both viscosity and pseudoplasticity of biofilms increased with agar concentration from 1.5 to 3 %. As it was more difficult to separate biofilms from 1.5 % agar plates due to softer agar we have grown biofilms for further experiments on 2 % agar plates. The trend of increasing biofilm viscosity with increasing agar concentration was clearly observed although the experimental uncertainty was rather high. To reduce experimental uncertainty we have increased the number of independent sample repetitions in subsequent biofilm experiments from $n = 3$ to $n = 11$ or higher.

Figure 3: Viscosity curve of *E. coli* biofilm (black) grown on different agar concentrations for 24 h. The average values and standard deviations are shown (n = 3).

Viscosity of biofilms from the wild type and mutant strains

Viscosity curves of different *E. coli* biofilms are given in Fig. 4. A significant difference in viscosity between different bacterial strain biofilms was observed. In particular, biofilm of *cla* mutant had the lowest viscosity in the entire range of shear velocity; i.e. the lowest viscosity curve. There was no significant difference in flow behavior between the wild type and *crl* mutant. To better characterize flow behavior we have determined the slope of the viscosity curves for different biofilms on log-log graphs, which is a proxy for a pseudoplasticity index. The slope was most negative for *wt* and *crl* mutant (-0.3 and -0.31 ± 0.05 , respectively) and was less so for *eps*-, *pga* and *cla* mutant, where it was -0.14 ± 0.02 .

Figure 4: Viscosity curves for *E. coli* MG1655 and its mutants grown on LB medium with 2 % agar, at 37 °C after 48 hours of incubation. Average values and standard error of the mean (n = 11 – 15) are given.

Viscoelasticity of wild type and mutants

Biofilm structures manifest a wide variety of mechanical responses, and they can exhibit numerous forms of self-organization whose description requires variables other than viscosity. One way to describe viscous and elastic behavior of biofilms is to measure viscoelastic curves. The results in Fig. 5 demonstrate the nonlinear viscoelastic nature of bacterial biofilm gel networks. The biofilm of the wild type strain had the highest storage modulus (G'), $\sim 10^5$ Pa, which indicates a strong gel structure. The viscoelastic response of mutant strains was significantly different from the wild type. All of the mutant strains had weaker biofilm gel stability. The strength of the biofilm structure as indicated by storage modulus G' for the wild type and mutant biofilms is given in Table 2.

Figure 5: Viscoelasticity curves for *E. coli* MG1655 and its mutants on LB growth medium with 2 % agar, at 37 °C after 48 hours of incubation. *cla* – magenta, *eps*- green, *pga* – red, *crl* – blue, *wt* – black. Average values and standard errors of the mean (n = 14 for wt and 15 for mutants) are given.

Alternatively, the binding or cohesive energy of a biofilm, a measure of the inter-molecular energy, can be derived. As given in Table 2 the cohesive energy was highest in the wild type strain, it decreased in *crl* mutant and was lowest in the *cla* mutant. Cohesive energy of biofilms per unit volume was reported to be in the range from 15 to 2700 pJ/μm³.⁶⁰ In our case the cohesive energy was from 1700 to 4200 pJ/μm³, which is slightly higher and consistent with high *G'* values measured for *E. coli* biofilms in this study. Based on the loss factor $\tan \delta$, which represents the ratio of the viscous and the elastic portion of the viscoelastic deformation (G''/G'), all biofilm structures show a degree of tack and stringiness at rest. This is consistent with the sticky nature of biofilm structures. The values of the loss factor $\tan \delta$ suggest that the wild type and *crl* mutant have biofilms with more a solid and brittle character than the other mutants.

Table 2: The average value of storage and loss modulus (G' and G'') at rest (at low shear strain deformation of 0.01 %), and standard deviation. The loss factor δ and cohesive energy at rest are given as well. Cohesive energy at rest was calculated as $\frac{1}{2} G' \times \gamma_c^2$.

Strain	G' [Pa]	G'' [Pa]	$\tan \delta = G''/G'$	Cohesive energy $\text{pJ}/\mu\text{m}^3$
<i>wt</i>	84000 ± 5000	19000 ± 1000	0.23	4200
<i>pga</i>	62000 ± 4000	17000 ± 1000	0.28	3100
<i>crl</i>	69000 ± 5000	17000 ± 1000	0.25	3400
<i>cla</i>	33000 ± 3000	9000 ± 1000	0.27	1700
<i>eps-</i>	40000 ± 5000	11000 ± 1000	0.27	2000

Due to nonlinear response to deformation the strength of the biofilm gel structure increased with increasing shear strain to the point of structure braking, as shown in Fig. 4. The shear strain at which biofilm gel structure starts to yield was highest for the wild type strain and decreased for *crl*, *cla*, *eps-* and *pga* mutant. The *pga* mutant biofilm structure yielded most easily. Compared to the wild type biofilm, the structure of the *pga* mutant yielded at approximately 50 % lower shear strain. With further increase of shear deformation, more structural damage occurred in the biofilm gel structure. From the shape of viscoelastic curves one may infer that the number and length of the cracks within the biofilm gel network increased with increased shear deformation. The structure of the biofilm gel network finally breaks at the flow point (the shear strain where $G' = G''$). This is also known as the gel/sol transition point. The yield point, shear strain at the point of collapse of biofilm structure, and flow transition index are given in Table 3. The results suggest that *pga* and *eps-* mutants were most pliable; they yield easily but break at high shear deformations. On the other hand, the structure of the wild type biofilm yielded last but flew first. The flow transition index, which indicates brittle or plastic behavior of the material, suggest that the structure of the wild type biofilm was the most brittle. The *pga* mutant, on the other hand, had the most plastic biofilm structure; although it yielded first it broke late.

Table 3: Shear deformation amplitude at the flow point when $G' = G''$. Flow transition index, defined as γ_f/γ_y (the ratio of the flow strain and the yield strain). The estimated experimental error is 15 %.

	$G' = G''$ [Pa]	Yield strain point [%]	Flow shear strain point [%]	Flow transition index
wt	10800	0.16	7.2	44
<i>pga</i>	6500	0.08	15.0	195
<i>crl</i>	7700	0.14	8.6	61
<i>cla</i>	5100	0.11	10.7	101
<i>eps-</i>	4800	0.09	16.9	190

Moisture effect on biofilm structure

It is expected that mechanical properties of biofilms grown at a solid-atmosphere interface are dependent on moisture content. In its natural environment of the intestine, *E. coli* biofilms are mostly exposed to solid/aqueous interfaces, although regular hydration/dehydration cycles may occur. To simulate hydration of the biofilms grown at a solid/air interface, biofilms were grown on agar plates and then submerged in water or surfactant solution for one minute. Next, the water was drained from the biofilms and their viscosity was measured. The results are given in Fig. 6. The addition of water dramatically changed the viscosity of the wild type and mutant biofilms. The viscosity dropped 10-fold or more. In addition, there was a significant change in the shape of the viscosity curves. Three distinct shear rate intervals could be observed in viscosity curves of biofilms after the addition of water or SDS solution. At low shear rates, the viscous curve approached a plateau of zero shear rate viscosity (η_0). This indicates that within this shear rate interval, the biofilm network structure breaks and reforms during the measurement interval. This results in only a small change of flow resistance in the biofilm structure with increasing shear rate. In the next shear rate interval, a shear thinning or pseudoplastic behavior is observed. Here the internal structures of the moist biofilm are deformed and oriented in the flow direction, which reduces internal friction in the biofilm. Therefore, the viscosity decreases with increasing shear rate. The slope was comparable to non-moistened biofilms. However, the pseudoplastic range upon deformation was much narrower in moistened biofilms. In the third shear rate interval, at high shear rates, the biofilm internal structures are thought to be maximally extended in the flow direction. The viscosity curve reaches an infinite shear viscosity (η_∞) and viscosity does not decrease with increasing shear rate.

The addition of 41 mM SDS solution to the biofilm had an effect on biofilm viscous properties. The viscosity drop was qualitatively comparable to the addition of water. In the wild type and *eps*-mutant the viscosity in SDS solution decreased more than in water. On the other hand, in *crl* and *cla* biofilms it decreased less than in water. There was no additional effect of added SDS in the *pga* mutant biofilm.

Figure 6: Viscosity curves of *E. coli* MG1655 wild type and mutants biofilms prior (black) and after addition of water (red) or 41 mM SDS (blue). (A: *wt*, B: *pga* mutant, C: *crl* mutant, D: *cla* mutant, E: *eps*-mutant, F: *B. subtilis* biofilm). Average values and standard errors of the mean (n=7–15) are given.

These results indicate that *E. coli* biofilms are hydrophilic, and can uptake water and thereby become soft. To check this interpretation using a well-studied control biofilm we grew *B. subtilis* biofilms, which are known to have very hydrophobic surfaces.^{43,51} The results after exposure to water and detergent solution are given in Fig 4F. There was a small drop of viscosity after addition of water or SDS solution. However, the decrease of viscosity upon water or SDS exposure was much less than in *E. coli* biofilms. In addition, there was no change of the shape of viscosity curves indicating that biofilm microstructure was not significantly different after adding water or SDS solution to *B. subtilis* biofilms, which is consistent with its hydrophobic nature.

The reduced viscosity upon hydration will lead to an increase diffusion, and potentially penetration of biocide into the biofilm, which was examined by adding hydrogen peroxide to moist biofilms. Biofilms were grown on glass slides for 48 h in LB liquid medium at 37 °C and treated with 6 % H₂O₂. The fractions of dead cells in different biofilms are shown in Fig. 7. In all cases, treatment with H₂O₂ increased the fraction of cells with compromised cytoplasmic membranes. H₂O₂ was significantly more effective in killing the *pga* mutant relative to the wild type strain. The effect of other extracellular compositions on the effectiveness of H₂O₂ treatments was not significantly different relative to the wild type.

Figure 7: The fraction of dead cells in biofilms treated with 6 % H₂O₂ as determined by Live/Dead test. Control samples (red) have been prepared in the same way as experimental samples (blue), but instead of H₂O₂ they were treated with phosphate buffer. Average values and standard errors of the mean (n=16-18) are given.

Discussion

Biofilm properties are dependent on the composition and structure of the extracellular polymeric material.¹⁷ Changing EPS composition has a significant impact on biofilm adhesion, formation, maturation and dispersion.⁶ However, there are only few studies addressing the effect of individual EPS components on biofilm viscoelastic properties.⁴⁴⁻⁴⁵ In this study, *E. coli* MG1655 was genetically modified to produce different extracellular matrix components which led to different mechanical strengths of the biofilms. The results suggest that there is a strong correlation between EPS composition and biofilm mechanical properties.

The viscosity was low in suspension cultures of *E. coli*. The bacterial suspensions had weak shear thinning behavior present only at low shear rates. On the other hand, as expected, the viscosity of the biofilms grown on agar surface was several orders greater. It is interesting to note that biofilm viscosity was dependent on the agar concentration. Biofilms were more viscous at increasing agar concentrations. The influence of the agar concentration on the dynamics of biofilms, viscosity, elasticity, intake of water and nutrients in biofilms have been noticed previously.⁴⁶ For example, Grant *et al.*⁴⁷ showed the relevance of the agarose concentration for the transition from planar to three dimensional biofilm topography in *E. coli*. All biofilms tested in this study were highly pseudoplastic, that is their viscosity decreased with increasing shear rate. Significantly, lower pseudoplasticity was observed in *cla* mutant, which overproduces colanic acid.

All biofilms formed strong viscoelastic gel structures, with different degrees of network strength. There are few reports in the literature of G' and G'' values for *E. coli* biofilms grown at solid-air interface. For example, Marinkova et al, 2015, studied the biofilms formed by *E. coli* and *P. fluorescens* during 5–17 h on several gel matrices and collected rheological data using TSM resonators. *E. coli* biofilm grown on these matrices had G' and G'' of 100 kPa and 400 kPa, respectively, at 15 MHz.⁶¹ On the other hand, Castro et al., in 2017 reported G' of 20 kPa and G'' of 100 kPa, at 15 MHz for biofilms formed on the gold electrode of quartz crystal resonators.⁶² In both cases, *E. coli* biofilms had higher moduli values compared to either *P. fluorescens* or *S. epidermidis* biofilms. Biofilms in the present study were tested at much lower frequency (1.6 Hz) and had G' in the range between 33 and 84 kPa, and G'' between 9 and 19 kPa. The strongest biofilm gel network structure was measured in the wild type and *crl* mutant strains. The latter

mutant expresses curli but not PGA, which suggests that curli are important for structural integrity in *E. coli* biofilms. Consistently, the *pga* mutant, which produces PGA but not curli, had significantly weaker biofilm structure. In addition, the biofilms of the *pga* mutant have lower viscosity and pseudoplasticity, less stable biofilm structure at rest, low yield shear deformation, but high flow transition index, which together makes these biofilms rather sticky. It has been shown that *pga* mutant produces very thick biofilms that are difficult to disperse, which has been attributed to the production of PGA.^{27,35,48} Based on the rheological data we infer that the reduced dispersion observed in this mutant may be due to relatively high cohesive energy and the ability of the *pga* mutant biofilm to spread easily before it breaks. The combined results for the *pga* and *crl* mutants suggest that the presence of curli dominates the wild type biofilm mechanical behavior. Curli are non-branched protein amyloid fibers with extensive β -sheet secondary structure. The rigid β -sheet structure gives the protein low flexibility, i.e. a high storage modulus, G' .

Biofilms of *cla* mutant have the lowest gel network strength at rest. This mutant does not produce PGA or curli, but it overproduces colanic acid. The biofilm structure yields at significantly lower shear deformations compared to the wild type, it has lower pseudoplasticity, a relatively high flow transition index and is tougher upon deformation. Colanic acid is a branched heteroglycan in the extracellular matrix of *E. coli* that is not significantly expressed in *wt E. coli* MG1655. In other *E. coli* strains, however, it allows for layered and less dense biofilm structures.⁴⁹ Beloin *et al.*³³ have shown that production of colanic acid, as a response to stress, decreases the formation of biofilms and its stability. In contrast, strains that do not produce colanic acid form densely packed biofilm structures.³¹ Lower viscosity observed in this mutant as well as low cohesive energy could be due to a looser biofilm structure and likely explains its lower biofilm stability.

Perhaps the most surprising result of this study is that *eps*- mutant which does not produce the major EPS polymers appears to be able to form stable biofilms on agar surface. In terms of its morphology this biofilm did not differ qualitatively from other biofilms. Nevertheless, its mechanical properties were different. The *eps*- biofilm had lower viscosity, lower pseudoplasticity, lower viscoelasticity, lower yielding point, higher flow transition point and low cohesive energy at rest compared to the wild type. These results suggest that *E. coli eps*- mutant produces extracellular substances that affect viscosity in the absence of PGA, curli and colanic acid biosynthesis, which are usually involved in biofilm formation. It seems likely that additional

EPS molecules are important for biofilm mechanical properties, such as LPS or other surface attached polymers.⁵⁰

Biofilms were grown on agar plates at the solid-atmosphere interphase. When such biofilms were exposed to water or a surface-active solution of SDS, the viscosity of biofilms decreased dramatically. This suggests that water was imbibed into the biofilm, which lubricated the biofilm internal structures and makes it substantially softer and less viscous. The effect of added SDS produced only a second order effect compared to the effect of water. In *crl* and *cla* mutants, which produce curli and colanic acid, SDS decreased viscosity less than the addition of water alone. This indicates that these biofilms are more polar than those of the wild type strain. On the other hand, in *eps*- mutant SDS increased the effect of water suggesting more hydrophobic interactions in this biofilm. All of these results are consistent with the polar nature of *E. coli* MG1655 biofilms, which allows relatively easy infiltration of water into the biofilm structure, which in turn softens biofilm structure. In sharp contrast, *B. subtilis* biofilm is at the other extreme. This biofilm is extremely hydrophobic and does not allow for an easy infiltration of water. It is known that surface cells of *B. subtilis* biofilms produce BslA protein, which makes biofilm surface superhydrophobic and affects biofilm mechanical properties.⁴³ Because the shape of the viscosity curve did not change upon addition of water or SDS, it can be inferred that the structure of this biofilm on the microscale did not change. The fact that SDS did not have an effect on superhydrophobic *B. subtilis* biofilms is consistent with other studies, wherein the addition of surface active agents, such as ethanol, had little effect up to 70 % ethanol concentration on the biofilm surface tension.⁵¹

Altered viscoelastic properties of mutant biofilms upon hydration could have an effect on biocide penetration and therefore its effectiveness in biofilms. Biocide penetration is directly dependent on the diffusion rate, which is inversely correlated to viscosity. To test this idea, biofilms were grown submersed on microscopic slides and treated with H₂O₂. After 48 hours of incubation, only the *pga* mutant formed thick biofilms, whereas other mutants and the wild type sparsely covered the surface with a relatively large fraction of dead cells indicating stressful conditions. The effect of added H₂O₂ was strongest in *pga* biofilm, where the fraction of viable cells was highest. It is possible that the larger fraction of viable cells made cells in biofilms producing PGA more

susceptible to biocide action. However, one should be careful to link biocide effectiveness in pga mutant directly to the PGA polymer as CsrA regulates many other genes unrelated to PGA.⁶³

In summary, the results of this study demonstrate the importance of EPS composition for biofilm mechanical properties. Biofilms of different species must confer properties that allow them to function appropriately under diverse environmental conditions, for example the hydrophobic properties of *B. subtilis* vs. the hydrophilic properties of *E. coli* biofilm likely relate to their roles in the soil vs. in the mammalian intestinal tract. Our findings demonstrate that viscoelastic properties of the biofilm can vary significantly in response to genetically altered expression of EPS components. Multiple factors and pathways participate in the genetic regulation of the EPS components of *E. coli*.^{24,38,52-54} Because cells have the means to modulate their EPS composition we propose that biofilm mechanical properties will change in accordance with environmental conditions. How the viscoelastic properties of the extracellular matrix specifically affect the various biological functions conferred by biofilms remains an important question to be answered.

ACKNOWLEDEMENT

This work benefited from the financial support of the Slovenian National Research Agency (P4-0116). No funding was received from industry.

REFERENCES

1. P. Stoodley, Z. Lewandowski, J. D. Boyle, and H. M. Lappin-Scott, *Biotechnol Bioeng*, 1999, **65**, 83-92.
2. V. Krstgens, H. C. Flemming, J. Wingender, and W. Borchard, *J. Microbiol Methods*, 2001, **46**, 9-17.
3. B. W. Towler, C. J. Rupp, A. B. Cunningham, and P. Stoodley, *Biofouling*, 2003, **19**(5).

4. T. Shaw, M. Winston, C. J. Rupp, I. Klapper, and P. Stoodley, *Phys Rev Lett*, 2004, **99**, 098102.
5. C. B. Whitchurch, T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick, *Science*, 2002, 295, 1487.
6. H. C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S. A. Rice, and S. Kjelleberg, *Nat Rev Microbiol*, 2016, **14**, 563-75.
7. H. Koo, M. L. Falsetta, and M. I. Klein, *J.Dent Res*, 2013, **92**, 1065-73.
8. B. W. Peterson, H. C. van der Mei, J. Sjollem, H. J. Busscher, and P. K. Sharma, *mBio*, 2013, **4**(5).
9. B. D. Ratner, A. S. Hoffman, F. J. Schoen, and J. Lemons, *In: Biomaterials Science: An introduction to materials in medicine*, 2004, 28–31.
10. C. Schaudinn, G. Carr, A. Gorur, D. Jaramillo, J. W. Costerton, and P. Webster, *J. Microsc*, 2009, **235**, 124-7.
11. Y. Asahi, J. Miura, T. Tsuda, S. Kuwabata, K. Tsunashima, Y. Noiri, T. Sakata, S. Ebisu, and M. Hayashi, *AMB Express*, 2015, **5**, 6.
12. A. Bridier, T. Meylheuc, and R. Briandet, *Micron*, 2013, **48**, 65-9.
13. P. K. Wallace, B. Arey, and W. F. Mahaffee, 2011. *Micron*, 2011, **42**, 579-85.
14. R.M. Donlan, and J.W. Costerton. *Clin Microbiol Rev*. 2002. **15**, 167–193.
15. D. Danilatos, and R. Postle, *Scan electron microsc.*, 1982, (Pt **1**): 1-16.
16. J. M. Vroom, K. J. De Grauw, H. C. Gerritsen, D. J. Bradshaw, P. D. Marsh, G. K. Watson, J. J. Birmingham, and C. Allison, *Appl Environ Microbiol*, 1999, **65**, 3502-3511.

17. N. Billings, A. Birjiniuk, T. S. Samad, P. S. Doyle, and K. Ribbeck, *Rep Prog Phys*, 2015, **78**, 3:036601.
18. P. C. Y. Lau, J. R. Dutcher, T. J. Beveridge, and J. S. Lam, *Biophys J.*, 2009, **96**, 2935-2948.
19. B. Stojković, S. Sretenovic, I. Dogsa, I. Poberaj, and D. Stopar, *Biophys J.*, 2015, **108**, 758-65
20. M. Wagner, D. Taherzadeh, C. Haisch, and H. Horn, *Biotechnol Bioeng*, 2010, **107**, 844-53.
21. B. W. Peterson, H. J. Busscher, P. K. Sharma, and H. C. van der Mei, *Microsc Microanal*, 2014, **20**, 912-5.
22. B. W. Peterson, Y. He, Y. Ren, A. Zerdoum, M. R. Libera, P. K. Sharma, A. J. van Winkelhoff, D. Neut, P. Stoodley, H. C. van der Mei, and H. J. Busscher, *FEMS Microbiol Rev*, 2015, **39**, 234-245.
23. I. Dogsa, M. Berloznik, D. Stopar, and I. Mandic-Mulec, *Plos One*, 2013, **8**, e62044 2013.
24. A. Parker, S. Cureoglu, N. De Lay, N. Majdalani, and S. Gottesman, *Mol Microbiol.*, 2017, **105**, 309-325.
25. A. Pannuri, H. Yakhnin, C. A. Vakulskas, A. N. Edwards, P. Babitzke, and T. Romeo, *J. Bacteriol.*, 2012, **194**, 79-89.
26. E. Wall, N. Majdalani, and S. Gottesman, *Annu Rev Microbiol*, 2018 **72**, 111-139.
27. X. Wang, J. Preston, and T. Romeo, *J Bacteriol.*, 2004. **186**, 2724–2734.
28. Y. Itoh, X. Wang, B. J. Hinnebusch, J. F. T. Preston III, and T. Romeo, *J. Bacteriol.*, 2005, **187**, 382-387.

29. Y. Itoh, J. D. Rice, C. Goller, A. Pannuri, J. Taylor, J. Meisner, T. J. Beveridge, J. F. Preston III, and T. Romeo, *J. Bacteriol.*, 2008, **190**, 3670-80.
30. D. R. Smith, J. E. Price, P. E. Burby, L. P. Blanco, J. Chamberlain, and M. R. Chapman, *Biomolecules*, 2017, **7**(4).
31. P. N. Danese, L. A. Pratt, and R. Kolter, *J. Bacteriol.*, 2000, **182**, 3593-3596.
32. D. H. Limoli, C. J. Jones, and D. J. Wozniak, *Microbiol spectr.*, 2015, **3**(3).
33. C. Beloin, A. Roux, and J. M. Ghigo, *Microbiol Immunol*, 2008, **322**, 249-289.
34. W. Thongsomboon, D. O. Serra, A. Possling, C. Hadjineophytou, R. Hengge, and L. Cegelski, *Science*, 2018, **359**, 334-338.
35. D. W. Jackson, J. W. Simecka, and T. Romeo, *J. Bacteriol.*, 2002, **184**, 3406-3410.
36. D. O. Serra, A. M. Richter, and R. Hengge, *J. Bacteriol.*, 2013, **195**, 5540-5554.
37. T. Romeo, and P. Babitzke, *Microbiol Spectr*, 2018, **6**(2).
38. T. Romeo, C. A. Vakulskas, and P. Babitzke, *Environ Microbiol.*, 2013, **15**, 313-24.
39. C. A. Vakulskas, A. H. Potts, P. Babitzke, B. M. M. Ahmer, and T. Romeo, *Microbiol Mol Biol Rev*, 2015, **79**, 193-224.
40. N. Navasa, L. Rodríguez-Aparicio, M. Á. Ferrero, A. Monteagudo-Mera, and H. Martínez-Blanco, *Biosci Rep*, 2013, **33**, 405-415.
41. P. Stefanic, and I. Mandic-Mulec, *J. Bacteriol.*, 2009, **191**, 1756-64.
42. S. Bas, M. Kramer, and D. Stopar, *Front. Microbiol*, 2017, **8**, 2443.
43. S. Kesel, S. Grumbein, I. Gümperlein, M. Tallawi, A. K. Marel, O. Lieleg, and M. Opitz, *Appl Environ Microbiol*, 2016, **82**, 2424-2432.

44. S. Chew, B. Kundukad, T. Seviour, J. R. van der Maarel, L. Yang, S. A. Rice, P. Doyle, and S. Kjelleberg, *mBio*, 2014, **5**:e01536-14.
45. P. Tielen, F. Rosenau, S. Wilhelm, K. C. Jaeger, H. C. Flemming, and J. Wingender, *BMC Microbiol*, 2013, **159**, 221–228.
46. D. R. Espeso, A. Carpio, and B. Einarsson, *Phys. Rev. E*, 2015, **91**, 022710.
47. M. A. A. Grant, B. Waclaw, R. J. Allen, and P. Cicuti, *J. R. Soc Interface*, 2014, **11**, 20140400.
48. D. W. Jackson, K. Suzuki, L. Oakford, J. W. Simecka, M. E. Hart, and T. Romeo, *J. Bacteriol.*, 2002, **184**, 290-301.
49. C. Prigent-Combaret, C. Prensier, T. T. L. Thi, O. Vidal, P. Lejeune, and C. Dorel, *Environ Microbiol*, 2000, **2**, 450-464.
50. R. Nakao, M. Ramstedt, S. N. Wai, and B. E. Uhlin, *PloS one*, 2012, **7**, e51241.
51. A. K. Epstein, B. Pokroy, A. Seminara, and J. Aizenberg, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 995-1000.
52. C. C. Goller, and T. Romeo, *Curr Top Microbiol Immunol*, 2008, **322**, 37-66.
53. R. Simm, I. Ahmad, M. Rhen, S. Le Guyon, and U. Römling, *Future Microbiol*, 2014, **9**, 1261-82.
54. S- L. Svenningsen. *Microbiol Spectr*, 2018, **6**(4).
55. Y. Wang, A. Pannuri, D. Ni, H. Zhou, X. Cao, X. Lu, and T. Romeo, Y. Huang, *J. Biol. Chem*, 2016, **291**, 10046-57.
56. P. J. Yang, M. LaMarca, C. Kaminski, D. I. Chuc, and D. L. Hu, *Soft Matter*, 2017,**13**, 4953-5088.

57. X. Wang, A. K. Dubey, K. Suzuki, C. S. Baker, P. Babitzke, and T. Romeo, *Mol. Microbiol.*, 2005, **56**, 1648-1663.
58. J. K. Schachterle, R. M. Stewart, M. B. Schachterle, J. T. Calder, H. Kang, and J. T. Prince, D. L. Erickson, *Front. Cell. Infect. Microbiol.*, 2018, **8**
doi: 10.3389/fcimb.2018.00323
59. C. Reichhardt, A. N. Jacobson, M. C. Maher, J. Uang, O. A. McCrate, M. Eckart, and L. Cegelski, *PLoS One*, 2015, 10(10):e0140388.
60. F. Ahimou, M. J. Semmens, P. J. Novak, and G. Haugstad, *Appl. Environ. Microbiol.* 2007, **73**, 2897–2904.
61. D. Marinkova, M. Michel, R. Raykova, D. Danalev, S. Yaneva, L. Yotova, and P. Griesmar, *Bulgarian Chem. Commun.* 2015, **47**, 436–444.
62. P. Castro, L. Elvira, J. R. Maestre, and F. M. de Espinosa, *Sensors*, 2017, **17**, 1395;
doi:10.3390/s17061395
63. A.H. Potts, C.A. Vakulskas, A. Pannuri, H. Yakhnin, P. Babitzke, and T. Romeo. *Nat Commun.*, 2017, 8, 1596. doi: 10.1038/s41467-017-01613-1.