# Supplemental Material (AEM 03273-15)

## Extended Material and methods

*Atomic force microscopy*

Atomic force microscopy is a common method to study mechanical surface properties of a given material at the nanoscale. Using soft cantilevers with spherical tips this method has also become applicable for soft materials such as bacterial biofilms (1, 2). A schematic of a typical AFM indentation experiment as performed here is given in **Fig. S1**. AFM measurements were performed using the contact mode; the soft cantilever carrying a bead at the cantilever tip was lowered vertically until brought in contact with the biofilm and then retracted again. Upon contact with the biofilm the cantilever bends, which is detected by a change in laser deflection at the PSD (position sensitive detection). With the spring constant of the cantilever known, this deflection signal can then be converted into a force signal.

With the obtained force curves, the surface stiffness of the bacterial biofilms can be determined. This stiffness (also called Young's modulus *E* or elastic modulus) represents the tensile stress divided by the tensile strain. For a soft cantilever with a bead at the cantilever tip, the Young's modulus *E* at a given Poisson ratio *v* (a material parameter describing the lateral expansion of an elastic material during compression, i.e. the ratio of transverse over axial strain) can be calculated with the Hertz model (3):

$$F = \frac{E}{1-\nu^2}\left[\frac{a^2+R^2}{2} ln \frac{R+a}{R-a} - aR\right]$$

with F the applied force on the cantilever, E the Young's modulus, R the radius of the bead at the tip, a the contact radius between the tip and the surface and $\nu$ the Poisson ratio.

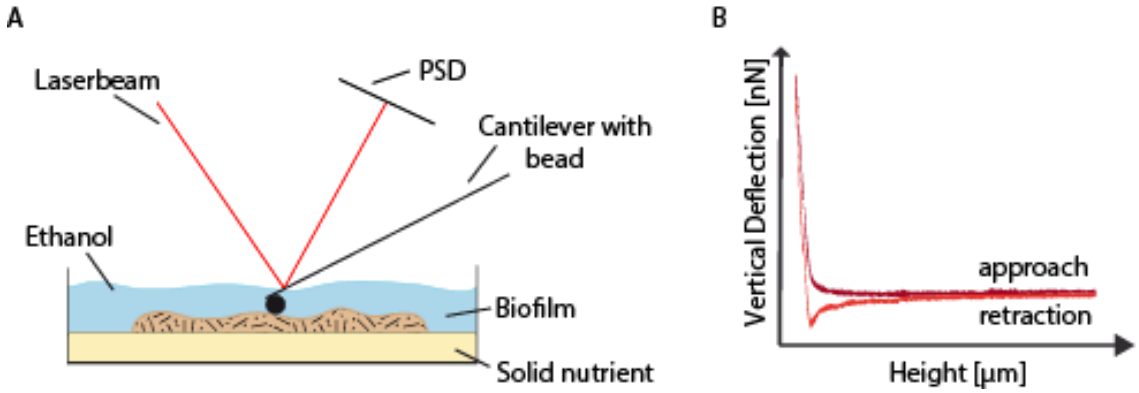


**Figure S1: Schematic representation of a surface indentation experiment on a bacterial biofilm using AFM.** A) One-day-old *Bacillus subtilis* biofilms were covered with 99% ethanol to minimize electrostatic interactions. B) Exemplary measurement data showing the cantilever deflection as a function of the distance between the cantilever and the biofilm surface (= height). The dark red curve represents the approach towards the surface and the light red curve depicts the retraction.

*Macrorheology*

Macrorheology is commonly used to determine the elastic and viscous properties of a given material. The material of interest is placed between two parallel plates (see **Fig. S2A**) where the lower plate is fixed. The upper plate is used to apply an oscillatory shear deformation to the sample and the torque necessary to deform the sample is measured. By inducing a sinusoidal shear deformation $\gamma$ (= strain),

$$\gamma(t) = \gamma_0 \sin(2\pi f t)$$

on the sample with an oscillation frequency *f* and measuring the stress response $\sigma$,

$$\sigma(\mathrm{t}) = \sigma_0 \sin(2\pi f t + \delta).$$

the elastic (= storage) modulus can be determined by $G'(f) = \frac{\sigma_0}{\gamma_0}\cos(\delta)$ and the viscous (= loss) modulus by $G''(f) = \frac{\sigma_0}{\gamma_0}\sin(\delta)$ where $\delta$ denotes the phase shift between the applied oscillatory deformation $\gamma(t)$, and the stress response $\sigma(t)$ (see Fig. S2B).

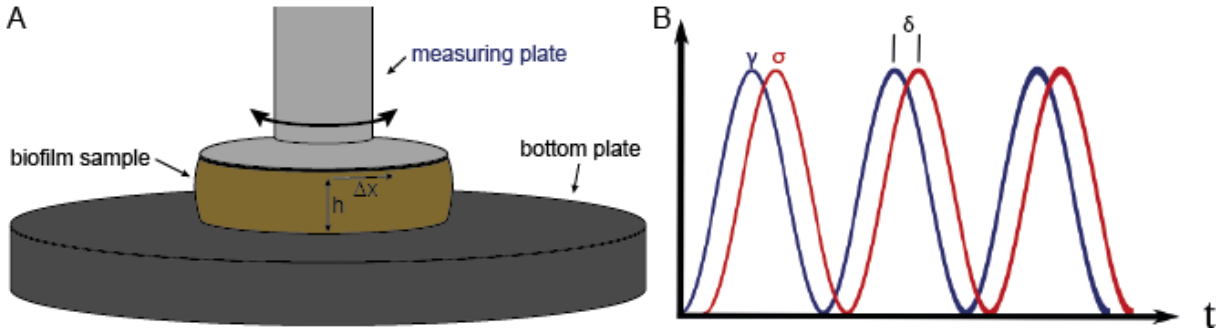


**Figure S2: Schematic representation of the measuring setup used for macrorheological characterization of a bacterial biofilm**. A) One day old biofilms were removed from agar plates by manual scraping, pooled and placed between two parallel plates of a rheometer. B) An oscillatory shear deformation (blue curve) is applied and the resulting stress (red curve) needed to deform the sample is measured. The phase shift between the deformation and the stress is used to calculate the viscoelastic moduli.

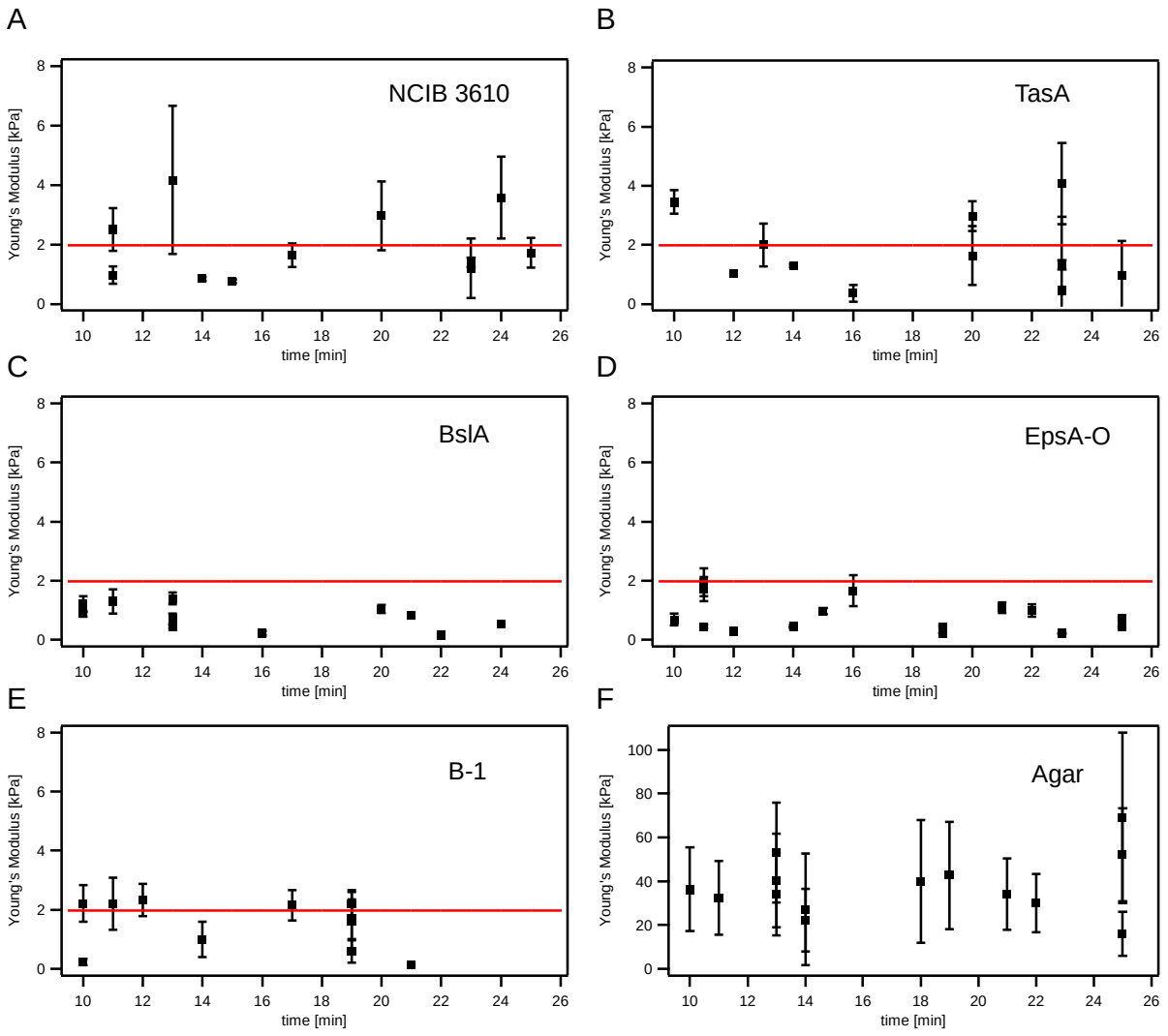


**Figure S3: Time distribution of data obtained by AFM nanoindentation.** As described in **Material and Methods** a minimum of ten minutes elapsed until the first AFM measurement could be performed. As the exact time-points of the AFM measurement then differed from sample to sample, we averaged over all data obtained for each strain in the time-window of 10-25 min to compare the data in an appropriate way. (A) NCIB 3610, (B) TasA, (C) BslA, (D) EpsA-O, (E) B-1, (F) data obtained for the surface of pure LB-Agar plates. Error bars denote the standard deviation of 64 single AFM nanoindentation experiments. The red line depicts the mean Young's modulus as obtained for the NCIB 3610 wild-type strain.

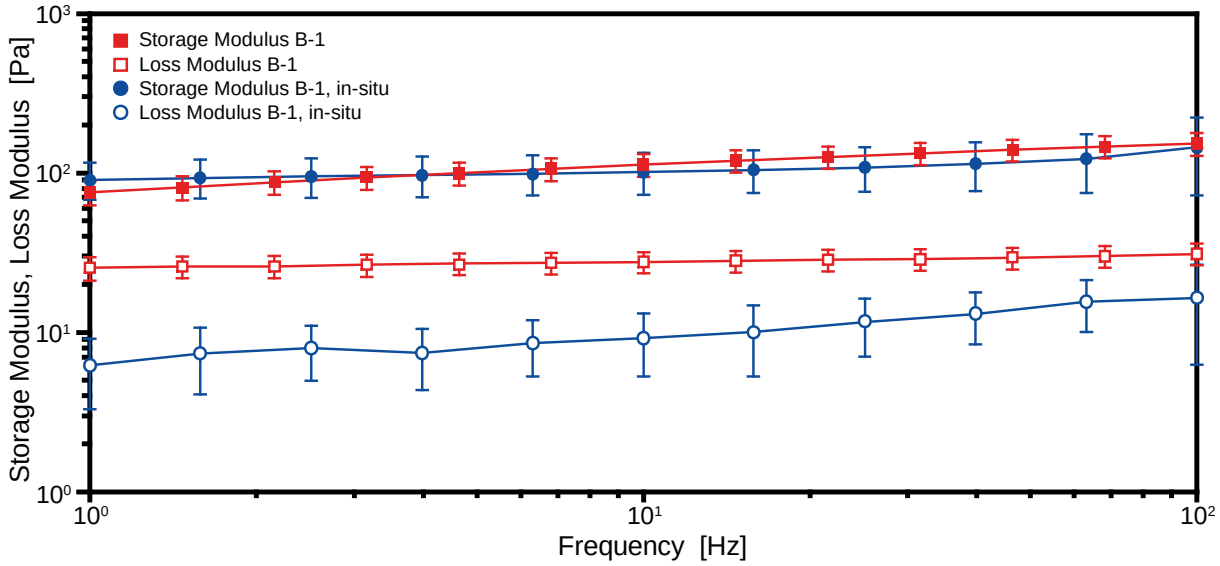


**Figure S4: Viscoelastic properties of *B. subtilis* B-1 biofilms grown in-situ.** Red squares depict the elastic moduli of biofilms grown on agar plates which were transferred to the rheometer fr mechanical evaluation as described in the Material and Methods section. Blue circles depict the elastic moduli of biofilms grown in-situ on the rheometer which match those of biofilms grown an agar very well. For this experiment, liquid media was filled in a custom built lower metal plate of the rheometer. This lower plate includes a reservoir for liquid media which is covered with a metal plate containing small holes (d=1 mm) to connect the top of the metal plate with the reservoir beneath. This allows for biofilm to grow at the upper surface of the plate where it can later be exposed to shear forces. At the same time, the bacteria growing on this metal plate are continuously provided with nutrients from the liquid media in the reservoir beneath. Biofilms were grown overnight at a temperature of 37 °C and formed comparable amounts of biofilm mass as when grown on agar plates. Error bars denote the standard deviation as obtained from at least 4 different samples of different growth batches.

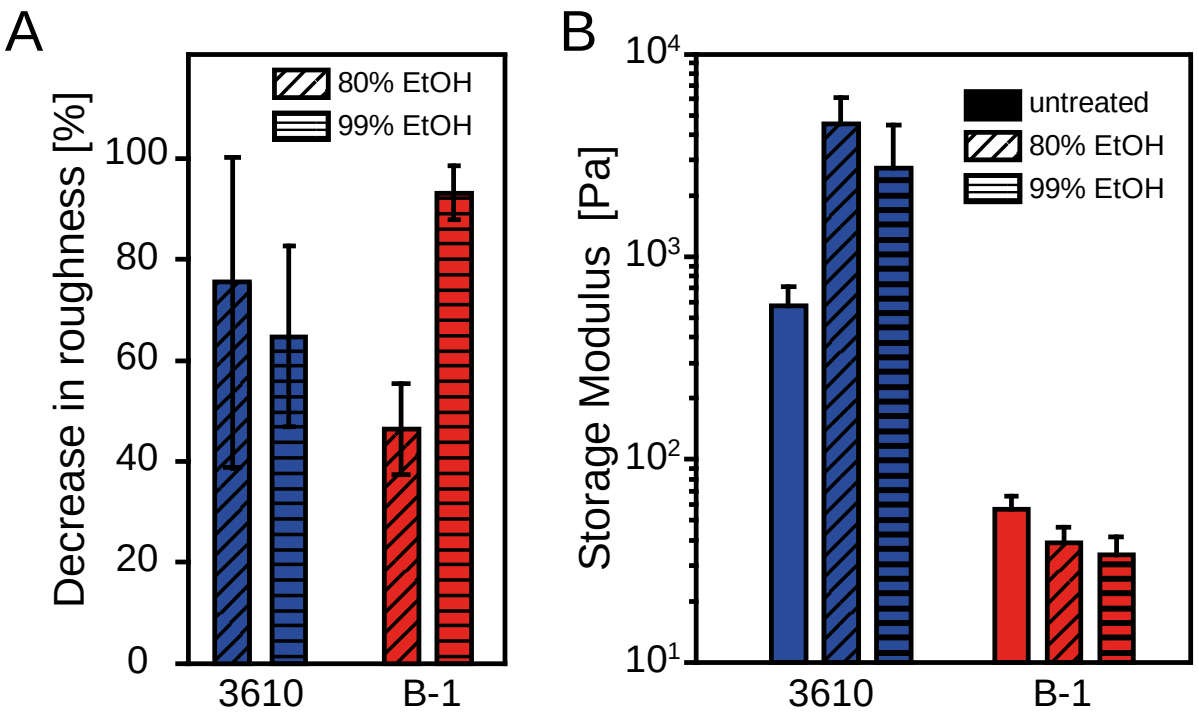


**Figure S5: Surface roughness and bulk elasticity of *B. subtilis* biofilms after ethanol treatment.** A) Decrease in roughness after treatment with 80% ethanol (diagonal lines) and 99% ethanol (horizontal lines) for 60 minutes. Virtually identical results are obtained for NCIB 3610 biofilms, whereas for B-1 biofilms the higher ethanol concentration entails a stronger decrease of the biofilm surface roughness. B) Elastic modulus of biofilms after treatment with 80% ethanol (diagonal lines) and 99% ethanol (horizontal lines) for 60 minutes. Both ethanol concentrations entail comparable experimental outcome, i.e. a stiffening of NCIB 3610 biofilms but no significant alteration for B-1 biofilms.

**Movie S1: Bubble formation by *B. subtilis* B-1 biofilms immediately after ethanol application.**

**Table S1: Significance analysis for data presented in Fig. 1.** The presented p-values are given in comparison to the data obtained for *ywsC* mRNA production. P-values below 0.05 depict significant differences in the tested gene mRNA production in comparison with the data obtained for *ywsC* mRNA production. The p-value for mRNA production of the *ywsC* gene at 10 hrs in comparison with the *ywsC* mRNA production at 18 hrs is 0.0033.

| Gene | p-values for data in Fig. 1a | p-values for data in Fig. 1b |
|---|---|---|
| *ywsC* | NA | NA |
| *tasA* | 4.2949e-15 | 3.659e-05 |
| *bslA* | 8.282e-15 | 0.00048 |
| *epsA-O* | 0.0035 | 0.7039 |

**Table S2: Significance analysis for data presented in Fig. 2-4.** The presented p-values are given in comparison to the data obtained for NCIB 3610. P-values below 0.05 depict significant differences of the corresponding data in comparison with the data obtained for strain NCIB 3610.

| Strain | p-values for data in Fig. 2 | p-values for data in Fig.3b | p-values for data in Fig. 3c | p-values for untreated data in Fig. 4a |
|---|---|---|---|---|
| NCIB 3610 | NA | NA | NA | NA |
| TasA | 0.7075 | 0.1928 | 0.7644 | 2.63E-06 |
| BslA | 0.0048 | 0.0289 | 0.0141 | 0.1120 |
| EpsA-O | 0.0016 | 0.0290 | 0.0056 | 0.0003 |
| B-1 | 0.2699 | 0.0002 | 0.0117 | 2.51E-12 |

**Table S3: Significance analysis for data presented in Fig. 4.** The presented p-values are given for the comparison of the data of the respective bacterial strain without application of ethanol. P-values below 0.05 depict that the data obtained in the presence of ethanol are significantly different from those obtained in the absence of ethanol for the respective strain.

| Strain | p-values 10 min ethanol Fig. 4a | p-values 60 min ethanol Fig. 4a | p-values 10 min ethanol Fig. 4b | p-values 60 min ethanol Fig. 4b |
|---|---|---|---|---|
| NCIB 3610 | 2.49E-12 | 8.08E-19 | 8.72E-11 | 2.95E-12 |
| NCIB 3610 + PGA | - | - | 0.4638 | 0.0149 |
| TasA | 5.17E-10 | 1.22E-16 | - | - |
| BslA | 1.80E-10 | 4.85E-16 | - | - |
| EpsA-O | 7.86E-15 | 1.38E-20 | - | - |
| B-1 | 0.2727 | 0.0032 | - | - |

**Table S4: Significance analysis for data presented in Fig. 5.** The presented p-values are given for the comparison of the data of the respective bacterial strain without application of ethanol. P-values below 0.05 depict that the data obtained in the presence of ethanol are significantly different from those obtained in the absence of ethanol for the respective strain. Grey: data obtained for total mass, white: data obtained for dried mass.

| Strain +- ethanol | p-values |
|---|---|
| NCIB 3610 | NA |
| NCIB 3610 + ethanol | 0.0011 |
| NCIB 3610 + ethanol + PGA | 0.3386 |
| NCIB 3610 | NA |
| NCIB 3610 + ethanol | 0.0043 |
| NCIB 3610 + ethanol + PGA | 0.2464 |
| B-1 | NA |
| B-1 + ethanol | 0.0037 |
| B-1 + ethanol + PGA | 0.9484 |
| B-1 | NA |
| B-1 + ethanol | 0.0175 |
| B-1 + ethanol + PGA | 0.1352 |

## References (Supplemental material only)


1. **Lau PC, Dutcher JR, Beveridge TJ, Lam JS.** 2009. Absolute quantitation of bacterial biofilm adhesion and viscoelasticity by microbead force spectroscopy. Biophys J **96:**2935-2948.
2. **Oh YJ, Lee NR, Jo W, Jung WK, Lim JS.** 2009. Effects of substrates on biofilm formation observed by atomic force microscopy. Ultramicroscopy **109:**874-880.
3. **Sneddon IN.** 1965. The relation between load and penetration in the axisymmetric boussinesq problem for a punch of arbitrary profile. Int J Engng Sci **3:**47-57.