Commonality of Elastic Relaxation Times in Biofilms

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Biofilms, sticky conglomerations of microorganisms and extracellular polymers, are among the Earth's most common life forms. One component for their survival is an ability to withstand external mechanical stress. Measurements indicate that biofilm elastic relaxation times are approximately the same (about 18 min) over a wide sample of biofilms though other material properties vary significantly. A possible survival significance of this time scale is that it is the shortest period over which a biofilm can mount a phenotypic response to transient mechanical stress.

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Microorganisms from all three domains of life—bacteria, archaea, and eukarya—demonstrate a propensity to attach and grow on surfaces in ubiquitous multicellular communities called biofilms, materials consisting of individual cells distributed within an exuded slime matrix of polysaccharides, proteins, and nucleic acids [1,2]. Biofilms appear in the fossil record in Precambrian marine stromatolite mats and in filamentous mats in ancient (3.235 BYA) hydrothermal vent environments [3]. They are also formed in modern hot spring and vent environments by species in the most deeply rooted lineages of archaea and bacteria, the Korarchaeota and *Aquificales* [4], suggesting that biofilm formation is an ancient and integral component of prokaryotic life. Today biofilms grow in virtually all aqueous environments under a wide range of mechanical shear forces ranging from quiescent ponds and lakes to the turbulent flows present in streams and industrial pipelines. Laboratory observations also demonstrate the dramatic ability of biofilms to adapt to changing shear stress environments [5]. Biofilm versatility is remarkable—the integrated combination of cells together with a protective polymeric matrix has proved a highly effective survival strategy.

One key component of this strategy is the ability to survive externally applied mechanical stress. Observations [6], rheometry [7,8], and measurements conducted on biofilm-agar mixtures [9,10] have suggested that biofilms behave as viscoelastic polymeric fluids, i.e., show elastic solidlike response to short time-scale stimuli and viscous fluidlike response to long time-scale stimuli. This dual behavior might provide a significant clue towards explaining biofilm robustness against environmental mechanical stress. Elastic materials absorb stress energy through deformation. Transient stress events might be resisted by reversible deformation. Long lasting stress could be dangerous, however—sustained storage of elastic strain (and hence elastic stress) risks structural failure. Viscous biofilm flow serves then to ease sustained internal stress by nonreversible deformation. The result is a rearrangement of biofilm so as to mitigate exposure to external shear stress. Other genetically modulated defenses may be possible as well, e.g., increased production of slime, and may, in fact, be necessary if external stress fluctuates more rapidly than biofilm flow can react. Such defenses are likely to be expensive, however, and are thus to be avoided if possible. These various considerations suggest that the time scale λ separating solid and fluid behavior is of interest. This crossover time between solid and fluid behavior, the elastic relaxation time, is the time over which temporary polymeric connections within the biofilm relax and conformational memory is lost.

A standard method for measuring material properties is the parallel plate rheometer creep test ([11], Chap. 5). In this test the material under study is placed between two circular parallel rheometer plates. A constant rotational torque τ_0 is applied to one of the plates over a given time interval $t \in [0, T]$. Displacement of the rheometer plate is measured and translated into material strain. For sufficiently small τ_0, the material responds linearly, and one of the following behaviors may be observed (Fig. 1): (a) An (idealized) elastic solid responds to an instantaneous stress at $t = 0$ by an instantaneous strain. Strain then remains constant until the removal of stress at $t = T$ at which time the material recoils to its original conformation. The elastic modulus G can be determined from the strain amplitude between $t = 0$ and $t = T$. (b) An (idealized) viscous fluid responds to a constant imposed stress on $t \in [0, T]$ by a linear in time strain. Displacement ceases when torque is released at $t = T$; there is no subsequent recoil. The fluid viscosity η can be determined from the slope of the strain curve. (c) An (idealized) viscoelastic fluid responds with characteristics of both elastic solid and viscous fluid behaviors. Application of an instantaneous torque results in immediate, reversible deformation as with an elastic solid. Over time, however, the material creeps irreversibly like a viscous fluid. Upon release of the applied torque at $t = T$ there is a partial recoil—initial conformation is partly or fully forgotten.

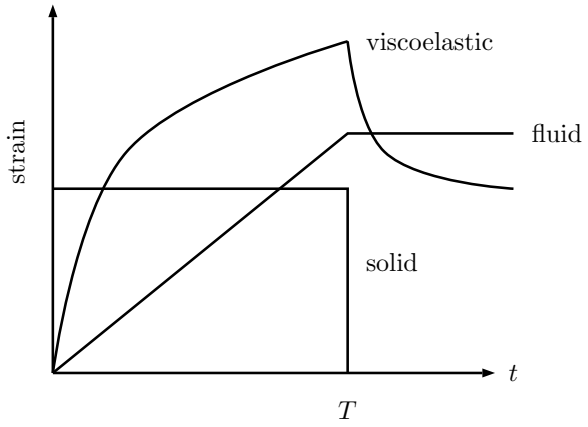

FIG. 1. Behavior of a viscoelastic fluid in response to applied stress. Consider a block of material, at rest prior to $t = 0$, to which a constant shear stress is applied over the time interval $t \in [0, T]$ after which time the applied stress is removed. Resulting response curves for idealized elastic solid, viscous fluid, and viscoelastic fluid are labeled. An (ideally) elastic solid responds at $t = 0$ with an instantaneous strain. The strain is reversed instantaneously at $t = T$; the deformation is temporary. An (ideally) viscous fluid responds with a linear in time shear flow until $t = T$ when the shear is removed. No recoil occurs; the deformation is permanent. An (ideally) viscoelastic fluid responds with a combination of the two behaviors, a reversible elastic strain and an irreversible viscous creep.

The time taken for irreversible deformation to entirely account for initial reversible deformation is λ, the elastic relaxation time scale.

We note that viscoelastic fluidity is a time-scale dependent phenomenon. A material subject to a creep experiment may exhibit a viscoelastic creep curve if the experimental time scale T is of the order of λ. However, the creep curve for that same material would resemble that of a viscous fluid if $T \gg \lambda$ and that of an elastic solid if $T \ll \lambda$. Note thus that characterization of a material as a viscoelastic fluid or not is a time-scale dependent decision determined by whether the ratio λ/T_0 (the Deborah number) of the elastic relaxation time scale λ to the observation time scale T_0 is $O(1)$ or not. Thus λ/T_0 is a fundamentally important parameter.

To determine λ, creep tests were performed on a wide variety of biofilms that were either grown directly on a flat plate of a parallel plate rheometer (as in [8]) or collected from the environment and then transferred to the plate. The particular biofilms tested were *Streptococcus mutans* UA159, a dental plaque colonizer, *Pseudomonas aeruginosa* FRD1, a pathogen isolated from the infected lung of a cystic fibrosis patient, *P. aeruginosa* PAO1, a nonmucoid clinical pathogen, a Nymph Creek (Yellowstone National Park) cyanobacteria biofilm [12], and a Chico Hot Springs (Montana) algal biofilm (the latter two are mixed species photosynthetic mats). In addition, data were included from a previous rheological study [7] performed on biofilms grown from pond water inoculum. For each type of biofilm a linear response regime (to torque τ_0) was estimated by checking the range of torques over which compliance (measured strain divided by applied stress) was approximately constant. All rheometer studies were performed within the estimated linear regime of the particular biofilm. Effective shear moduli G were calculated from the biofilm strain response to an applied shear stress and effective viscosities η were calculated from subsequent biofilm creep (Fig. 2). (In addition to the data collected from

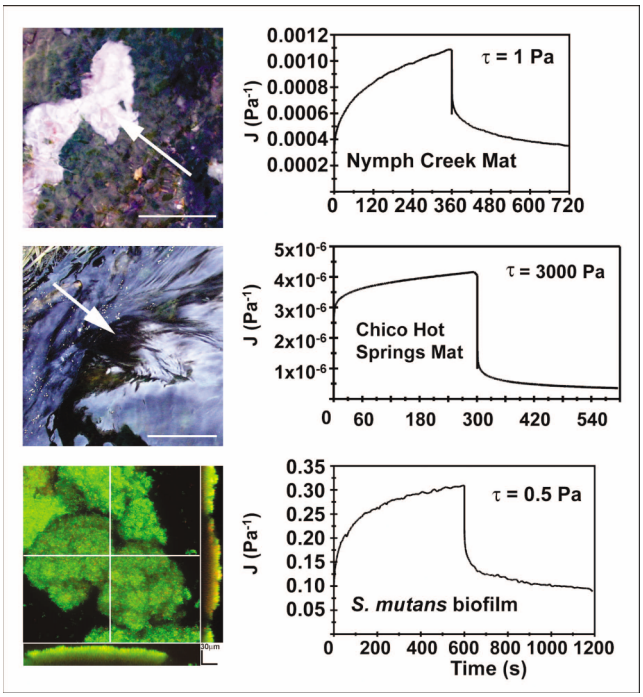

FIG. 2 (color online). Representative examples of creep test data. Compliance J (measured strain/applied stress) is plotted versus time. Here "applied stress" refers to the constant stress applied during the initial creep period of the experiments. Top: photograph of a cyanobacterial biofilm collected from Nymph Creek, Yellowstone National Park, together with creep curve. Applied stress was 1 Pa for 6 min followed by 6 min of recovery. Middle: photograph of an algal biofilm collected from Chico Hot Springs, Montana, together with creep curve. Applied stress was 3000 Pa for 5 min followed by 5 min of recovery. Bottom: Confocal micrograph of a *S. mutans* biofilm grown on a 20 mm hydroxyapatite coated rheometer plate (plan view is 1×1 mm^2, cross sections through vertical and horizontal planes are 1 mm long and 0.2 mm in height) together with creep curve. Applied stress was 0.5 Pa for 10 min followed by 10 min of recovery. All show characteristic viscoelastic fluid response with similar elastic relaxation times. Viscosities η were calculated from the inverse asymptotic slope normalized by stress during the applied stress period. Shear moduli G were calculated from the strain displacement (minus the flow displacement) normalized by stress during the applied stress period.

rheometer experiments, some additional data were also included from observations of structure deflection in flow cells [13] to measure effective shear moduli and effective viscosities for *S. aureus* biofilms as well as some of the *P. aeruginosa* PAO1 and FRD1 biofilms. In these cases determination of the linear regime is difficult.) Here "effective" refers to the fact that biofilms are nonhomogeneous materials both with respect to internal structure and to conformational geometry. Hence parameter measurements should be regarded as effective values for the biofilm considered as a whole material including heterogeneities. G versus η for 44 biofilms is plotted in Fig. 3. A very wide range of material parameter values were observed; effective shear moduli and effective viscosities each ranged over eight decades. The wide range is not necessarily a surprise; one can expect large natural variability among significantly differing biofilms with significant variation in growth environments and histories, and the studied biofilms are, indeed, dramatically dissimilar in many respects. However, one important property of viscoelastic materials was observed to vary much less, namely, the elastic relaxation time. λ was estimated to be the time required for viscous creep length to equal elastic deformation length (so that memory of initial conditions is lost), i.e., $\lambda \approx \eta/G$. (In fact, for a given material in many instances it is useful to measure a spectrum of relaxation times—we refer here to the longest such time.) A least squares line fit of the log-log data in Fig. 3 results in the best fit $\log\eta = 1.03\log G + 3.04$, with result $\eta/G^{1.03} \approx \bar{\lambda} \approx 1100$, approximately 18 min. (For comparison purposes, we note that elastic relaxation times of aggregates of embryonic chicken cells have been measured to be approximately 0.5 min [14].) Individual values of λ vary relatively little compared to the material

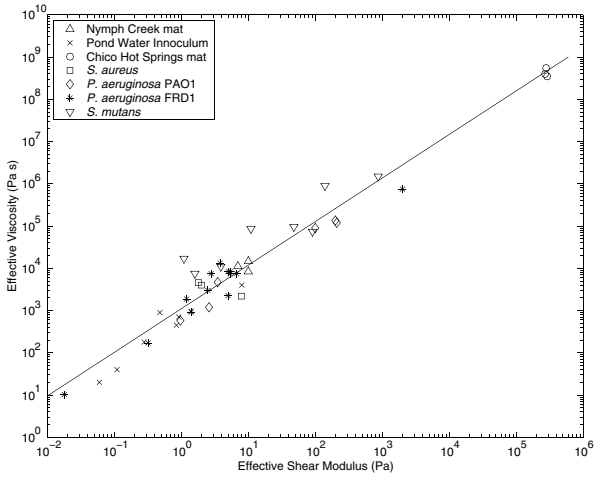

FIG. 3. Plot of effective shear modulus vs effective viscosity for 44 tested biofilms. The straight line is $\log\eta = 1.03\log G + \log\bar{\lambda}$, the least squares best fit. $\bar{\lambda} \approx 1100$ s is the best fit for the elastic relaxation time.

parameters η and G among the studied biofilms as well; λ was measured to lie within a range 3.5×10^2 to 2.6×10^3 s in all cases. Some of the variability in η and G is probably a result of natural biofilm heterogeneity and variability. In particular, it should be noted that biofilm coverage in rheometer coupons was generally not uniform, and thus biofilm contact with the rheometer was not generally uniform. Partial contact can be expected to result in underestimation of both G and η with the error factor approximately equal to the proportional area of biofilm-rheometer contact. Microscopy studies indicate that biofilm-rheometer contact was generally well over half of the rheometer disk area. Further, this partial contact area should largely cancel out of the ratio η/G, and hence we believe it has little significant effect on calculated values of λ. In the case of the flow cell observations, applied shear stress at the biofilm-bulk fluid interface was estimated from the bulk flow rate. The resulting error in calculated values of η and G is difficult to quantify, but, again, this error should be proportionally the same in η and G; thus, we expect it will approximately cancel in the ratio η/G. λ is a time scale and measurements should be relatively insensitive to spatially dependent errors.

Biofilms are found in widely different varieties and environments and also exhibit widely different properties. Hence convergent behavior, when it occurs, must necessarily be suspected to have critical survival impact. One such convergent property is the viscoelastic transition, the elastic relaxation time scale for a given material that distinguishes solid from fluid response to mechanical stress. What is the significance of the commonality of $\lambda \approx$ 18 min? Interestingly, this is the same time scale as that of the measured phenotypic response at the cellular level to changes in chemical levels in the environment [15,16]. For successful and persistent colonization of surfaces in flowing environments, biofilms must be able to adapt to fluctuations in mechanical stresses. We can hypothesize that the viscoelastic response of biofilms afford a buffering time during which cells within the biofilm can generate an adaptive phenotypic response to prevent catastrophic detachment. Molecular processes such as protein folding and binding interactions are much faster (milliseconds), and prokaryotic development sequences such as fruiting body development in myxobacteria [17] or biofilm development in *P. aeruginosa* [18] are much slower (days). On intermediate time scales it is possible that a biofilm can increase the strength of its structural matrix phenotypically in response to mechanical stresses by, for example, increasing extracellular polymer production. Changes in alginate production in response to environmental stress have been observed in *P. aeruginosa* biofilms approximately 1 h after onset [19]. (Planktonic cells can respond somewhat faster with time scales ranging from approximately 5 min up to hours, e.g., [20–22].)

An interesting and effective strategy combining mechanical and genetic response is thus suggested. In ancient environments, biofilms developed defenses to enable persistence on surfaces while maintaining close proximity to the nutrient rich flowing vent waters and holding the organisms spatially in a stable homeostatic growth environment. These same mechanisms may today be utilized by biofilms to withstand mechanical stresses and persist on the surfaces of modern man-made industrial and medical components as well as eukaryotic tissue. In particular, to avoid prolonged exposure to mechanical stress in any environment, biofilms must be able to deform in response or risk catastrophic structural failure. However, overly fast deformation might, counterproductively, result in structural failure through washout. Likewise, overly slow deformation may prematurely trigger expensive genetically modulated reaction to shear stress or may even result in growth processes overtaking deformation. Hence the biofilm elastic relaxation time should be shorter than the biological response time to allow structural deformation, but otherwise as long as possible.

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