Rheology of living cells

R. Portela, J.M. Franco, P.L. Almeida, P. Patrício, R.G. Sobral and C.R. Leal

Abstract — **The mechanical behavior of living cells, during planktonic growth, has been thoroughly explored combining common biological techniques with rheology and rheo-imaging measurements. Under a shear flow, bacterial cultures of *Staphylococcus aureus* revealed a complex and rich rheological behavior not usually accessed in biological studies. In particular, in stationary shear flow, the viscosity increased during the *exponential phase* and returned close to its initial value at the *late phase* of growth, accompanied by the stabilization of the bacterial population. In oscillatory flow, the elastic and viscous moduli exhibited power-law behaviors whose exponents are dependent on the bacteria growth stage, and can be associated to a Soft Glassy Material behavior. These behaviors were framed in a microscopic model that suggests the formation of a dynamic web-like structure, where specific aggregation phenomena may occur, depending on growth stage and cell density. Furthermore, systematic measurements combining optical density and dry weight techniques presented new evidences, which confirmed that the observed cell aggregation patterns developed during growth, under shear, can not only be cell density dependent.**

I. Introduction

In this work we briefly describe the full set of experimental and theoretical results obtained during the characterization of living bacterial cultures, when subject to a shear flow. This study focused on *Staphylococcus aureus* cultures during growth and used rheological techniques to access their mechanical properties. One of the main objectives of this study was to understand the observed complex and rich rheological behaviors revealed by these bacterial cultures along growth [1-3]. It is now clear that the observed behaviors can be associated with cell density and aggregation patterns that are developed during culture growth, showing a cell collective behavior.

In previous works, it was experimentally described that, at a certain time lapse during the *exponential phase* of growth, when subjected to a simple shear flow, the viscosity augmented abruptly and recovered to close to the initial value. This comportment has no equivalent in the bacterial growth curve measured by optical density monitorization. On the other hand, in oscillatory flow, the elastic and viscous moduli presented power-law behaviors whose exponents were related with the bacterial growth phase. Such power-law dependencies may be described within the Soft Glassy Material model [4] framework. Moreover, these behaviors can be interpreted via a microscopic model that considers the development of a dynamic web-like structure, where jamming phenomena may occur, depending on growth stage and cell density [1,2]. The establishment of such web-like assemblies at a particular time interval throughout bacterial growth was possible to be detected, by combining rheology with novel observations carried out in a different rheometer, where real-time image acquisition was also performed along with viscosity measurements [3]. More recently, systematic measurements of optical density and dry weight, accomplished during culture growth have brought new evidences to this study, namely that the bacteria growth process is always in the diluted regime, in the considered time window, in which the cell density increases linearly.

In the following, the main results of this study will be presented along with the experimental procedures in the Methods, Results and Discussion section and the final remarks presented in the Conclusions.

II. Methods, Results and Discussion

A. Bacterial culture

The studied bacterial system was the human pathogen *Staphylococcus aureus*, considered as a study model due to its coccoid cell shape, regular morphology and clinical relevance: methicillin-resistant *Staphylococcus aureus* (MRSA) strain COL [5] was chosen. The bacterial cultures were grown in erlenmeyer flasks (1000 ml total volume) in an orbital shaker incubator with agitation (180 rpm) and the initial volume of the culture was of 200 mL [1].

B. Optical density, Colony forming units and Dry weight determination

The growth monitoring was performed in 96 well plates with a volume of culture of 200 µL per well and the apparatus was set to maintain the temperature at 37ºC and measure the OD_{620nm} at discrete elapsed times, resorting to a microplate spectrophotometer Ultrospec 2100 pro.

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In parallel, we also determined the population's colony forming units (cfus/mL), which provides an estimate of the number of viable cells, by plating serial dilutions of the bacterial cultures on tryptic soy agar (TSA), incubating for 48 h at 37ºC, and counting the colonies.

For the determination of the bacterial dry weight, DW, 200 mL of fresh LB were inoculated as before. Along the growth curve, for the optical density values indicated in the results, samples of 10 mL of each culture were taken. The cells were harvested at 9000xg and the supernatant discarded. The cells were then washed in the same volume of phosphate buffer 10 mM, pH 7. The cell pellets were dried at 90ºC during 24h assuring that no trace of buffer was still present. DW measurements has an associated error of 0.1 mg/mL.

The results obtained from OD_{620nm} and cfus/mL measurements allowed to establish the time intervals of each growth stage for the *S. aureus* culture: *lag phase* for t≤300 min; *exponential phase* for 300≤t≤450 min and *late phase* for t>450 min (±30 min).

Considering the bacteria density close to the water density (1 g/mL), the DW can be directly related with the cell volume fraction, ϕ, at each time point by $DW = 10^3\phi$. This allowed to establish a relation between the optical density values and ϕ, showing two new evidences: i) the growth process occurs at very low volume fraction values and may be considered in the diluted regime, since $\phi \leq 0.02$ [6] and ii) it is possible to say that the relationship between the optical density and ϕ is linear as $OD_{620nm}=3159.6\phi$ (given by the best linear fit), presented in Fig.1. In Table 1, the main results obtained in this section are presented.

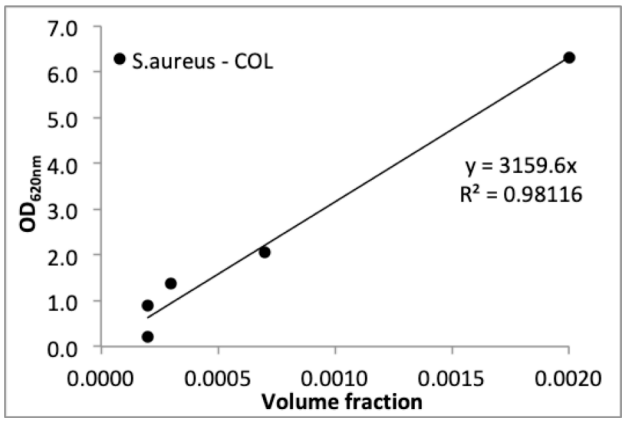

Figure 1: Optical densities, OD_{620nm}, as a function of cell volume fraction for *S. aureus* – strain COL cultures during growth. All measurements were performed at 37 ºC. Solid line represents the best fit to the experimental data.

C. Optical microscopy

Optical microscopy, using a Leica DMR microscope associated to a Leica DFC320 camera and Leica IM500 Image software V1.20, was performed to characterize the aggregation processes during growth. For each aliquot, at specific growth moments, 250, 350, 410 and 550 min, a calibrated volume of sample of 10µL was observed. 10 photos were randomly taken. From these images the bacteria distribution during growth was evaluated, namely the number of clusters vs the number of bacteria per cluster and the average number of bacteria per cluster vs time [2].

The studied *S. aureus* culture presented the formation of an increasing number of clusters during growth, although the average number of bacteria in each cluster (observed in the microscope image plane), #b/c, is not higher then 3±1, see Table 1.

D. Rheology and Rheo-imaging

Rheological measurements were performed in a controlled stress rotational rheometer Bohlin Gemini HRnano. A steel plate/plate geometry, with diameter 40 mm and 2000 µm gap (to ensure a good signal), was used for the measurements of the viscosity growth curve, at a constant shear rate of 10 s^{-1} (which mimics the incubator conditions), during 1200 min over the same culture sample. Measurements were performed at 37ºC to allow optimal bacterial growth. The viscosity of LB culture medium was also measured in identical conditions and presents an average value of 0.32 ± 0.22 mPa.s.

A steel cone/plate geometry, with a diameter of 40 mm, an angle of 2º and 70 µm gap, was used to perform oscillatory measurements. We measured the elastic and viscous moduli, G′ and G″, respectively in function of the angular velocity, ω, in the linear regime, imposing 10% of strain. Assays were performed at 20ºC, to refrain bacteria growth during measurements. A solvent trap was used in all measurements to avoid evaporation.

Representative curves of the described rheological properties for the *S. aureus* – strain COL culture are presented in Fig. 2 and 3. The steady-state shear viscosity growth curve, measured at a constant shear rate of 10 s^{-1} is represented in relative values in Fig. 2.

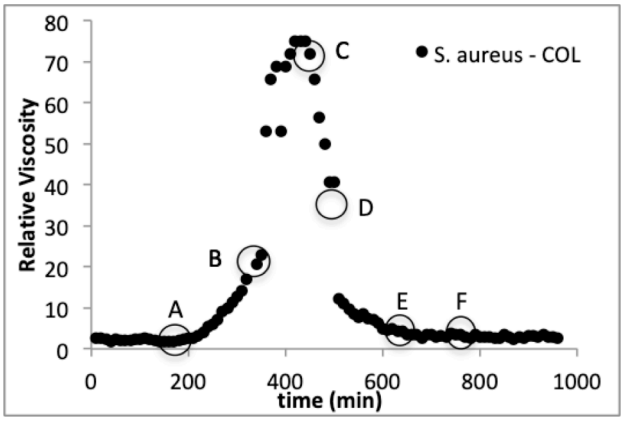

Figure 2: a) Steady-state shear viscosity growth curve of a *S. aureus* - COL culture, measured at a constant shear rate of 10 s^{-1}, in relative values η/η_0, where η_0 is the culture medium viscosity; measurements were performed at 37ºC; Letters A – F indicate the time intervals for which images were taken during rheo-imaging experiments.

An increase of ~70x, with respect to the medium culture viscosity value was observed to occur during the *exponential phase*. It was followed by an almost immediate recovery to the initial relative viscosity value, during the *late phase*. The

main features of these results were also reproduced during the rheo-imaging measurements [3] although in a different experimental set-up (equipment and geometry).

Table 1: Optical and rheological properties of *S. aureus* – COL culture characterized at specific time points during growth: number of bacteria per cluster (observed in the microscope image plane), #b/c; optical density, OD620nm; dry weight, DW (mg/mL); volume fraction, ϕ; theoretical values of the power-law exponent, *x*, and tan$\pi x/2$; experimental value of G''/G' and η/η_0.

TABLE I. OPTICAL AND RHEOLOGICAL PROPERTIES

	Time (min)					
	250	***350***	***410***	***475***	***550***	***1400***
#b/c	3	2	2	-	2	-
OD_{620nm}	0.22	1.37	2.07	-	-	6.32
DW	0.2	0.3	0.7	-	-	2
ϕ	0.0002	0.0003	0.0007	-	-	0.002
x	~0.80	~0.79	-	~0.23	~0.39	-
tanπx/2	3.1	2.9	-	0.4	0.8	-
G''/G'	~2.4	~2.2	-	~0.6	~0.9	-
η/η_0	5.9	22.8	71.9	56.3	7.8	2.6

For the oscillatory flow measurements, the elastic and viscous moduli, G' and G'', exhibited similar power-law behavior, at some interval with respect to the angular velocity, see Fig. 3. It was also observed that the power-law exponents were dependent on the bacteria growth stage [2].

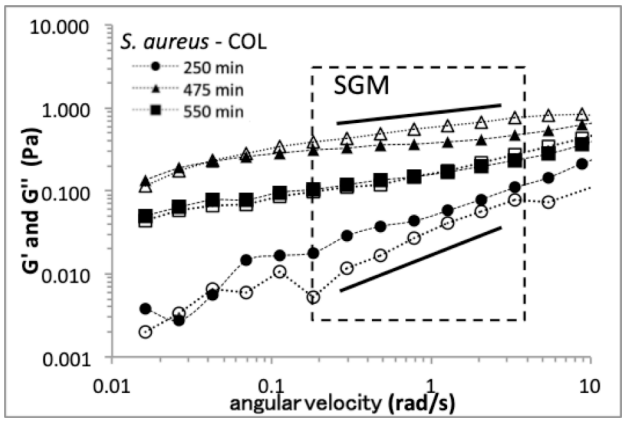

Figure 3: Oscillatory shear flow: elastic modulus G′ (open symbols) and viscous modulus G″ (full symbols), in function of the angular velocity, ω, for *S. aureus* – COL culture aliquots with 250, 475 and 550 min of growth time. Dashed box defines the identical power-law dependence of G′ and G″ with ω as described by the Soft Glassy Materials model. Solid lines are guidelines that correspond to specific power laws. All measurements were performed at 20ºC (to diminish bacteria growth through measurements).

These behaviors are well described by the Soft Glassy Material (SGM) [4] model, where the elastic and the viscous moduli present the same weak power law dependence on the angular velocity and can be written as $G' \sim \omega^x$ and $G'' \sim \omega^x$, respectively, from which G''/G'~tan$\pi x/2$ is obtained. The exponent *x* represents an effective noise-temperature factor, which takes values in the range $0<x<1$. When $x=0$ the model describes the ideal solid case – a perfect elastic body; when $x=1$ it describes the ideal liquid case – a perfect viscous fluid system; and when $0<x<1$ the system has an intermediate behavior and may flow. In Table 1, the angular frequency exponent values of *G'* and *G''* are compared with the SGM model predictions. A good agreement between experimental measurements and theory was obtained.

Real-time image acquisition was performed during steady-state shear flow measurements in a Haake RheoScope equipment, which combines the principles of a conventional controlled-stress rheometer with an optical microscope. A constant shear rate of 10 s^{-1} was imposed using cone-plate geometry with a diameter of 70 mm and 1º and a gap of 25 μm, at 37 ºC. The cone had a mirror surface and the plate a cover glass, to allow optical microscopic observations (20x) during shear, at an intermediate radius plate fixed position. Video image acquisition was performed during 150 min. A frame image was selected from the video at each minute. In these tests, the growth of a *S. aureus* culture was followed starting measurements already at the *exponential phase* (approximately at an OD_{620nm}=2.5). The original video, will be included to this work as supplementary material, from which representative images were extracted and were included in Fig. 4.

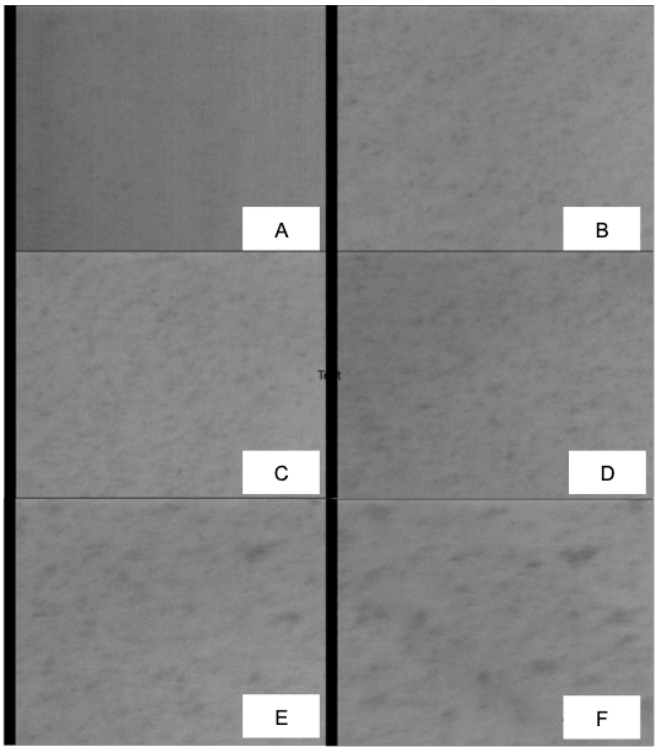

Figure 4: Real-time images acquired during steady-state viscosity growth curve in rheo-imaging measurements of a *S. aureus* – COL culture, with correspondent times to η/ηo curve represented in Fig. 2; in images collected at B, C and D instants, jammed structures can be identified; in images collected at E and F instants, cell aggregates can be observed (dark areas), corresponding to cell sedimentation at the fixed bottom glass-plate of the RheoScope. Images width: 100 μm. The original video, from where these images were extracted, will be included in this work as supplementary material.

The formation of a web-like structure can be clearly observed in the time interval corresponding to images B – C, in the *exponential phase*: the bacteria presented a dynamic self-organization where wave rows were formed, with dozens of cells, like cell strings. This structure could only be maintained during the *exponential phase* and started to collapse as the bacterial culture approached the *lag phase*.

For longer times, within the *late phase* of growth, a cell deposition process was initiated and small bacteria collections sedimented, leading to the dark regions in images E and F (this last one showing more and bigger aggregates created) in Fig. 4. This phenomena provides an elucidation to the viscosity decline in the *lag phase* of growth: although the number of cells existing in the culture was elevated, the cells were not able to maintain themselves in suspension in the culture medium.

III. Conclusions

We have observed the formation of web-like structures, based on cell strings that resemble cell necklaces, at a specific time interval during the *exponential phase* of the bacteria growth, followed by sedimentation and subsequent enlargement of bacterial aggregates, in the *stationary phase*. These findings, along with the evidence that the growth process occurs always in the diluted regime, were essential to corroborate our microscopic model previously proposed [1,2] and to establish the relevance of the adhesion factors at specific time intervals during the bacteria growth, in particular during the *exponential phase.*

Appendix

The original video, Saureus-COL.m4v, from where the presented images in Fig.4 were extracted, will be included in this work as supplementary material.

Acknowledgment

Strain COL and Strain RUSAL9 were a kind gift from H. de Lencastre and A. Tomasz. This article is based upon work from COST Action MP1305, supported by COST (European Cooperation in Science and Technology). This work was also supported by FEDER through COMPETE 2020; FCT Projects No. UID/CTM/50025/2013 and PTDC/FIS-NAN/0117/2014 (P.L.A.); ESCMID Grant No. 2015 (R.G.S.); and the Unidade de Ciências Biomoleculares Aplicadas - UCIBIO, which is financed by national funds from FCT/MEC (Grant No. UID/Multi/04378/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement No. POCI-01-0145- FEDER-007728.

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