


BRIEF REPORT

Yeast Biofilm as a Bridge Between Medical and Environmental Microbiology Across Different Detection Techniques

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ABSTRACT

Medical and environmental microbiology have two distinct, although very short, histories stemming, the first from the pioneering works of Sommelet, Pasteur, Lister and Koch, the second mainly from the studies of Beijerinck and Winogradsky. These two branches of microbiology evolved and specialized separately producing distinct communities and evolving rather different approaches and techniques. The evidence accumulated in recent decades indicate that indeed most of the medically relevant microorganisms have a short circulation within the nosocomial environment and a larger one

involving the external, i.e. non-nosocomial, and the hospital environments. This evidence suggests that the differences between approaches should yield to a convergent approach aimed at solving the increasing problem represented by infectious diseases for the increasingly less resistant human communities. Microbial biofilm is one of the major systems used by these microbes to resist the harsh conditions of the natural and anthropic environment, and the even worse ones related to medical settings. This paper presents a brief outline of the converging interest of both environmental and medical microbiology toward a better understanding of microbial biofilm and of the various innovative techniques that can be employed to characterize, in a timely and quantitative manner, these complex structures. Among these, micro-Raman

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along with micro-Brillouin offer high hopes of describing biofilms both at the subcellular and supercellular level, with the possibility of characterizing the various landscapes of the different biofilms. The possibility of adding a taxonomic identification of the cells comprising the biofilm is a complex aspect presenting several technical issues that will require further studies in the years to come.

Keywords: Biofilm; *Candida*; Environmental microbiology; Medical microbiology; Micro-Brillouin; Micro-Raman; Yeasts

INTRODUCTION

Several opportunistic fungi are often found in food, environmental substrates and medical settings [1]. Of these, *Aspergillus* is probably one of the best known [2], although the problem involves several yeast species that can produce both exogenous and endogenous nosocomial infections [3]. The latter are carried out by strains already present in the hospital and propagating from one patient to another, possibly surviving on surfaces, devices and even operators' hands [4, 5]. Although the isolates from hospitals appear to be more virulent than others [6], hospital conditions and therapies can produce relatively rapid selections producing a significant microevolution toward increased resistance to drugs [7] and to detergents [8]. One of the key factors in the circulation of yeasts from the environment to the patient, and potentially vice versa, is the biofilm. This structure is well known to impart increased resistance to the cells with a variety of mechanisms [9, 10].

In this paper, we propose the concept that biofilm is one of the most important factors to ensure the survival and also the dissemination of the fungal cells throughout different habitats, including those of medical and clinical environments. At the same time, we propose a novel way of characterizing biofilm through its metabolomics and mechanical characteristics, aiming at understanding their role in their stability on surfaces and the conditions allowing the delivering of large amounts of cells for the colonization of other surfaces. This article does

not contain any studies with human participants or animals performed by any of the authors.

OPPORTUNISTIC YEASTS ARE PRESENT IN DIFFERENT ENVIRONMENTS AND FOOD

The idea that pathogenic and opportunistic species are typical of medical and clinical settings is well confirmed for species such as *Candida albicans* which can cause both endogenous and exogenous infections [3]. In contrast, other species can be isolated from both food and environmental samples. Probably the best known cases are the presence of *Cryptococcus neoformans* in pigeon droppings [11]. *C. gattii* has been found in soil, tree surfaces, air, and water in throughout the year [12]. *Meyerozyma guilliermondii* (formerly *Candida guilliermondii*), a species widely present in food [13] and on fruit [14], is also found in clinical settings. It has also been shown using different markers that fruit isolates are rather different from those deriving from environmental and clinical samples, outlining a kind of ongoing speciation [15]. *M. guilliermondii* has been found in cheese in association with *C. parapsilosis*, which is also frequently found on environmental samples together with its sibling species, *C. orthopsilosis* and *C. metapsilosis* [16]. In a recent unpublished survey, we found large amounts of *C. parapsilosis* on fruits in the Mediterranean area (Cardinali, personal communication).

Together, this evidence suggests that not only pathogenic bacteria [17] and molds [18] are isolated from food and environmental samples but also a large number of yeast species. Considering the expanding realm of pathogenic fungi, these observations suggest that even more environmental and food-borne yeasts will be likely enumerated among opportunistic species in the years to come.

BIOFILM IS CRITICAL IN ENSURING THE SUCCESS OF THE SPECIES

Invasive fungal infections cause more than 1.6 million deaths worldwide every year with a

burden similar to tuberculosis, yet are the most understudied group of pathogens in microbiology [19]. The ability to produce biofilm is one of the risk factors for mortality in patients [20], and one of the most effective mechanisms for the resistance to drugs [20]. The mechanisms of the resistance of fungal planktonic and sessile cells are quite complex and numerous [21]. Among them, the outstanding efficacy of efflux pumps is being actively studied [22], whereas less is known about the mechanism, role and positioning within the

biofilm of the “persister” cells. The situation is further complicated by the large variability due to the presence of five prominent species, in addition to *C. albicans* [23]. Moreover, the many recent cases caused by *C. auris* has led to the considering of this yeast as a worldwide threat and has enlarged the set of highly dangerous opportunistic yeasts of the genus *Candida* [24].

We have recently demonstrated that biofilm production is the key factor in the success of opportunistic yeasts in the hospital

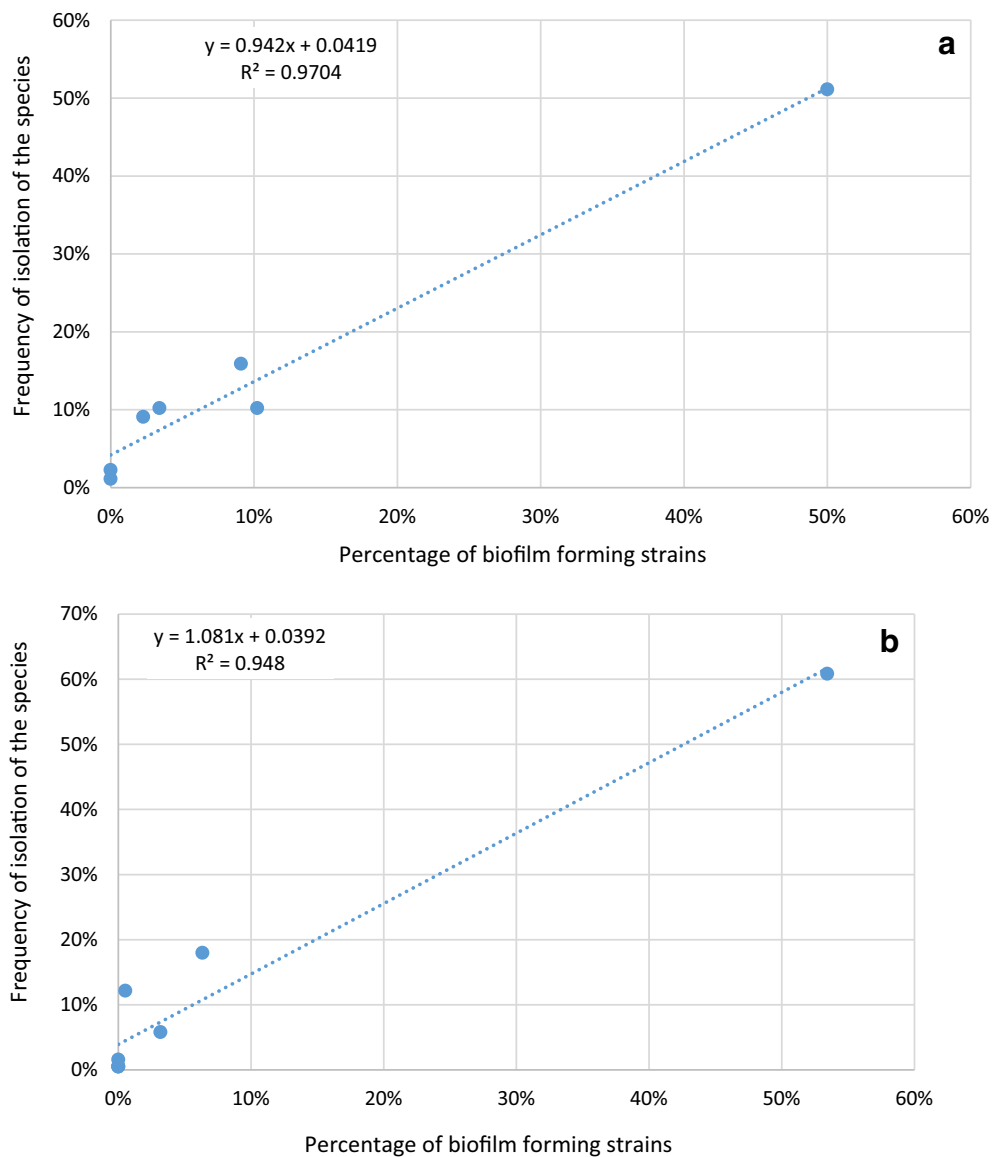


Fig. 1 Regression between the percentage of biofilm forming strains and the frequency of isolation: **a** data from Pisa Hospital; **b** data from Udine Hospital

environment [25]. In fact, a linear relationship has been found between the percentage of biofilm-forming strains within each species and the frequency of isolation of that very species in the hospital (Fig. 1). Even more interestingly, the regression functions obtained with the isolates at the Pisa and Udine Hospitals in Italy are rather different (Fig. 1a vs. b) and both have a strong support ($R^2 = 0.97$ and 0.95 for Pisa and Udine, respectively). This experimental evidence suggests that the permanence and spreading of yeast biofilms may be due to intrinsic characteristics, either of the specific hospital or of the biofilm structure of different strains and species. The second hypothesis calls for the setting up of an ad hoc system to evaluate the mechanical properties that could be instrumental in the ability of biofilms to remain attached to surfaces even in the presence of mechanical and chemical stresses such as those caused by the process of surface cleaning.

A NEW CHARACTERIZATION OF MICROBIAL BIOFILM, THROUGH ITS MECHANICAL PARAMETERS

In order to meet the need for an accurate system to determine the mechanical properties of yeast biofilm, a set-up was recently realized to perform Brillouin–Raman micro-spectroscopy measurements [26] for the mechanical mapping with chemical specificity of biological samples [27–30] (Fig. 2). The Brillouin spectrum, with a peak at a frequency shift of about 18 GHz, shows the mechanical (viscoelastic) properties, while the relative intensities of the Raman peaks show the chemical composition inside the scattering volume. The square of the Brillouin frequency shift is proportional to the longitudinal elastic modulus, while the linewidth of the Brillouin peaks is related to the viscosity of the sample inside the scattering

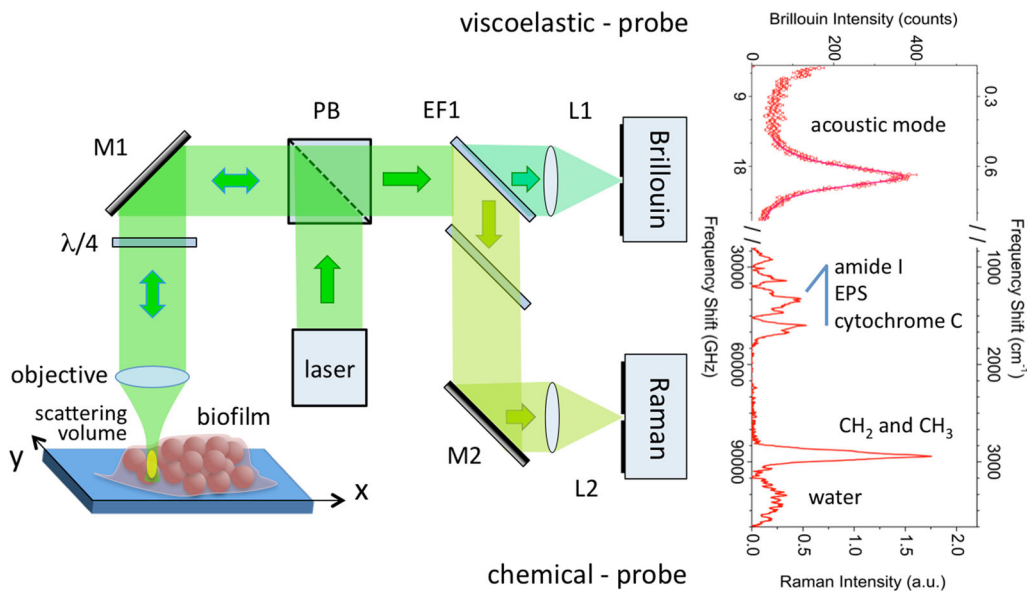


Fig. 2 Schematic of the set-up for Brillouin-Raman micro spectroscopy measurements [26], with (right side) typical Brillouin and Raman spectra from one single point of the microfilm. Light from a laser source is reflected by a polarizing beam splitter (PB) into a $\times 50$ microscope objective lens and focused through a $\sim 1 \mu\text{m} \times 1 \mu\text{m}$ area of the sample. The light back-scattered by the (few micrometers thick) scattering volume is split by an edge

filter (EF1), the high-frequency-shift component is sent to the Raman monochromator and the low-frequency shift is sent to the Fabry–Perot interferometer. The sample is mounted on top of a xyz translation stage for 1- and 2-D mapping of the mechanical and chemical properties of the sample. 3-D mapping is also possible, in the case of transparent samples. More details are reported in Ref. [26]

volume [28, 30]. In addition, when the thickness of the sample is lower than that of the scattering volume, the intensity of the Brillouin peak is a measure of the thickness of the sample (the thicker the sample, the more scattered the light).

LINEAR MAPPING OF A *C. ALBICANS* BIOFILM

The morphology of the *C. albicans* biofilm grown over an aluminum substrate is visible in the photomicrograph of Fig. 3. Brillouin and Raman spectra have been recorded along a 20- μm linear region (green line in Fig. 2), with 1- μm steps. The intensity, frequency shift and width of the Brillouin lines are reported in the righthand panels of Fig. 2, showing, in the central region of the scan, a $\sim 6\%$ reduction of Brillouin frequency together with an increase by a factor of 2 in the linewidth. These features are correlated with an increase by a factor of 3 of the intensity, I_0 , suggesting that a three-layer structure of the biofilm exists in the central region. The joint Raman investigation (lefthand panels in Fig. 3) shows an order of magnitude increase of water content in the same region. On this basis, the combined reduction of stiffness and static viscosity can be attributed to the plasticizing effect associated with a local increase of residual water, as previously observed in dehydrated tissues [28]. These results (the larger the thickness, the more plastic the behavior, and the more intense the Raman signals) can be consistently interpreted in terms of a region in which a chemico-physical condition is preserved that is favorable to the survival of *Candida* cells. This hypothesis is reinforced by the corresponding increase of Raman signals in the 1510- to 1750- cm^{-1} region, which can be, at least partially, attributed to the resonant scattering from cytochrome c, a marker of cell vitality [31, 32]. This result is in line with the microbiological evidence that the biofilm acts as a structure which increases the resistance of the cells to challenging agents.

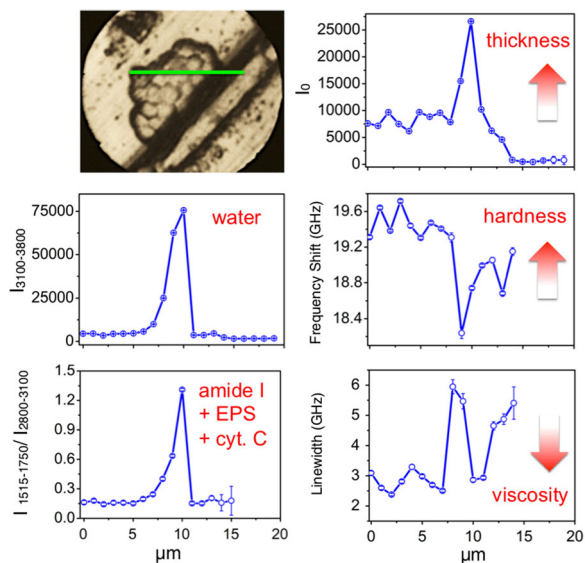


Fig. 3 Photomicrograph and results of the analysis of Brillouin and Raman spectra of *C. albicans* on an aluminum substrate [26, 34]. The green line denotes a 20- μm -long region where Brillouin and Raman spectra were collected, using a 1- μm step size. Righthand panels Brillouin intensity I_0 , frequency shift and linewidth, proportional to thickness, hardness and viscosity, respectively. Lefthand panels Raman integrated intensity of the OH region (3100–3800 cm^{-1}), and of amide I + EPS constituents + cytochrome c (1515–1750 cm^{-1}) normalized to the intensity of the CH_2 and CH_3 stretching region (2800–3100 cm^{-1})

2D MAPPING OF *C. PARAPSILOSIS* BIOFILM

A *C. parapsilosis* biofilm grown on stainless steel was analyzed by our combined spectroscopic technique. Brillouin and Raman spectra have been recorded along a 6 $\mu\text{m} \times 6 \mu\text{m}$ map, with 1- μm steps in both directions (yellow square frame in Fig. 4a). The intensity of Brillouin lines (Fig. 4b) shows a maximum, twice the average, in the bottom right corner, indicating a two-layer morphology in that region. In this case, the maximum of residual water (Fig. 4f) is not located inside the buried cell but inside a cell participating in the single layered border of the biofilm. The 1515- to 1750- cm^{-1} Raman intensity (Fig. 4e), comprising the cytochrome c signal, is maximum in the same region, suggesting, as in the case of *C. albicans*, the

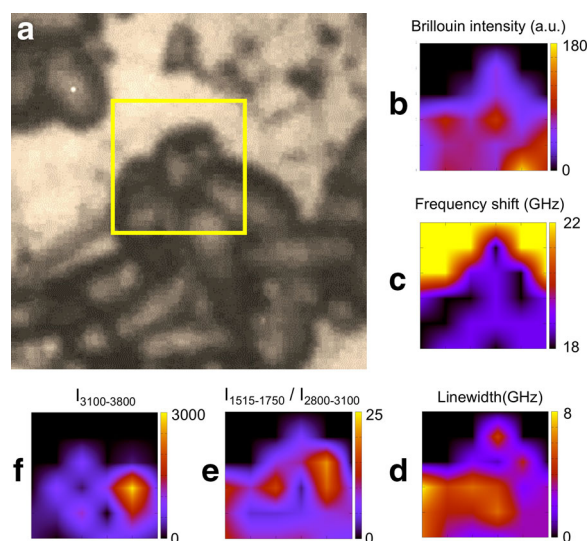


Fig. 4 Photomicrograph and results of the analysis of Brillouin and Raman spectra of *C. parapsilosis* grown on stainless steel. **a** Yellow box a $6\ \mu\text{m} \times 6\ \mu\text{m}$ region where Brillouin and Raman spectra were collected, using a $1\text{-}\mu\text{m}$ step-size in both directions. **b** Brillouin intensity, I_0 , **c** frequency shift and **d** linewidth. Raman integrated intensity: **e** of amide I + EPS constituents + cytochrome c ($1515\text{--}1750\ \text{cm}^{-1}$) and **f** of the OH region ($3100\text{--}3800\ \text{cm}^{-1}$)

presence of a cell which has survived to drying. In addition, the maximum of the $1515\text{--}1750\text{-cm}^{-1}$ Raman signal extends in a larger region, revealing the presence of the EPS component. In fact, superimposed on the micrograph, Fig. 4e is more intense in the interstitial region between yeast cells. The minimum of the Brillouin frequency shift and the maximum of the linewidth correlate well with both the more hydrated region and the region reaching more to the EPS, both of them corresponding to a softer viscoelastic behavior.

CONCLUSION

Opportunistic yeasts are a serious threat to public health; their presence and circulation involves various environments and food beyond the medical settings. One of the key factors governing the success of these organisms is their ability to produce biofilms, complex structures with peculiar mechanical features. A micro-

Raman coupled with a micro-Brillouin device, developed in our laboratories, was able to characterize some of these mechanical characteristics and will be used in future to define whether they have strain- or species-specific variability. These results corroborate previous findings of our working groups [26, 33], and similar results, although limited to just the Brillouin technique, have been obtained with the bacterium *Pseudomonas aeruginosa* [34]. Taken together, these findings indicate that the present technique has a huge potential in unravelling the mechanical properties of the complex structure of both bacterial and fungal biofilms.

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Compliance with Ethics Guidelines. This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability. The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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