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Three-dimensional characterization of bacterial microcolonies on solid agar-based culture media

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ABSTRACT

For the last century, *in vitro* diagnostic process in microbiology has mainly relied on the growth of bacteria on the surface of a solid agar medium. Nevertheless, few studies focused in the past on the dynamics of microcolonies growth on agar surface before 8 to 10 h of incubation. In this article, chromatic confocal microscopy has been applied to characterize the early development of a bacterial colony. This technology relies on a differential focusing depth of the white light. It allows one to fully measure the tridimensional shape of microcolonies more quickly than classical confocal microscopy but with the same spatial resolution. Placing the device in an incubator, the method was able to individually track colonies growing on an agar plate, and to follow the evolution of their surface or volume. Using an appropriate statistical modeling framework, for a given microorganism, the doubling time has been estimated for each individual colony, as well as its variability between colonies, both within and between agar plates. A proof of concept led on four bacterial strains of four distinct species demonstrated the feasibility and the interest of the approach. It showed in particular that doubling times derived from early tridimensional measurements on microcolonies differed from classical measurements in micro-dilutions based on optical diffusion.

Such a precise characterization of the tri-dimensional shape of microcolonies in their late-lag to early-exponential phase could be beneficial in terms of *in vitro* diagnostics. Indeed, real-time monitoring of the biomass available in a colony could allow to run well established microbial identification workflows like, for instance, MALDI-TOF mass-spectrometry, as soon as a sufficient quantity of material is available, thereby reducing the time needed to provide a diagnostic. Moreover, as done for pre-identification of macro-colonies, morphological indicators such as three-dimensional growth profiles derived from microcolonies could be used to perform a first pre-identification step, but in a shorten time.

1. Introduction

Microorganisms found in clinical and industrial settings require accurate identification to assist in patient therapy, manufacturing processes or environmental monitoring. Growth of bacteria on the surface of a solid agar medium still represents one of the key first steps microbiologists apply within the diagnostic process. Bacterial growth leads to the appearance of visible colonies containing clonal bacterial cells. Although the number of cells within a colony is species-dependent, each visible colony usually contains at least one million cells. The shape, color and growth rate of a colony can already assist in the identification process (Singleton et al., 2004; Matsushita et al., 2004).

Microcolonies are in the late-lag phase to early-exponential phase. They appear within a few hours on or under the surface of the agar plate and their diameters are usually smaller than 350 μm (London et al., 2010; Goodwin et al., 2006). Microcolonies have already been considered for accelerated bacterial identification. Early on, pictures showing morphological characteristics of microcolonies were indeed used for early identification attempts (Hadzieva and Hadziev, 1987). More recently, digital imaging of autofluorescent colonies (Growth Direct system, Rapid Micro Systems, Bedford, USA) was introduced to provide an early enumeration of pathogens on an agar plate (London et al., 2010). Alternatively, a microbial high-throughput screening instrument based on elastic light scattering patterns allowed rapid identification of microcolonies (Bae et al., 2011). Several studies using confocal microscopy have also contributed to a better understanding of microcolony development. For instance, Su et al. (2012) focused on an *Escherichia coli* strain and reported that a two-dimensional

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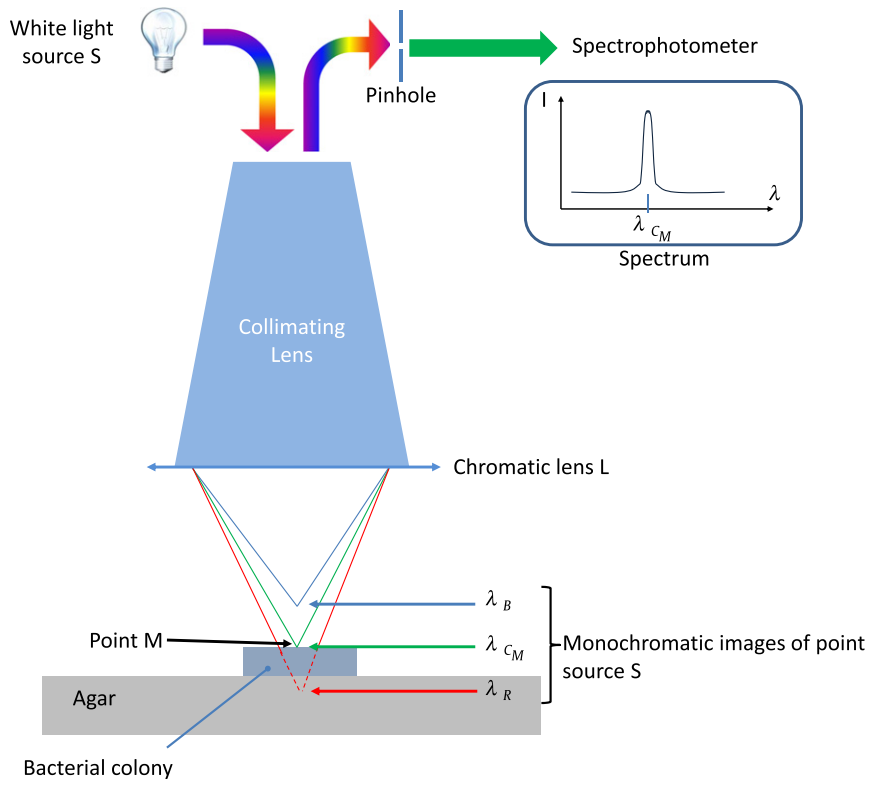

Fig. 1. Schematic view of the chromatic confocal sensor. A point source *S* of white light is differentially focused by an extended axial chromatic lens *L*. Hence, a given wavelength (from blue to red, λ_B to λ_R) is associated to a focusing depth. The backscattered light from the point *M* on the top of the colony located in the light path enters a pinhole filtering all wavelengths except λ_{C_M}. The collected light with intensity *I* is then analyzed by a computer-controlled spectrometer from which the elevation of the point *M* is derived.

expansion of microcolonies preceded a three-dimensional growth phase. More recently, Kim et al. (2013) described a confocal displacement meter combined with optical density circuits that allows one to characterize the height, diameter and optical density of a bacterial microcolony.

We present here a new method for the rapid characterization of bacterial microcolonies based on chromatic confocal microscopy. This technique, introduced in early 1980s (Molesini et al., 1984), and developed in the 1990s relies on a differential focusing depth of the spectrum of wavelengths of a white source lamp by means of a chromatic lens. Compared to classical confocal microscopy, it allows to quickly scan a surface without any vertical movement of the optical device, while maintaining the same spatial resolution (Chun et al., 2009). This approach was applied to four strains of distinct bacterial species. The resulting surface and volume kinetics were used to estimate the growth rates of microcolonies at the early stage of growth, and to study its variability within and between agar plates.

2. Material and methods

2.1. Strains preparation and growth kinetics measurements by microdilution

A set of four strains (*Enterococcus faecalis* ATCC 51299, *Pseudomonas aeruginosa* ATCC 10145, *Proteus mirabilis* ATCC 35659 and *Staphylococcus epidermidis* ATCC 49741) was selected. In order to determine their planktonic doubling time, microbial suspensions (0.5McF = 10^8CFU · mL^{-1}) were prepared from bacteria grown on Columbia agar plates with 5% sheep blood (COS) (bioMérieux, Marcy l'Étoile, France) and incubated at 35 °C for 24 h. These suspensions were then diluted in a saline solution to a concentration of 10^6CFU × mL^{-1} for *S. epidermidis* and 10^4CFU · mL^{-1} for the other strains. *S. epidermidis* is known to grow slowly and considering the limit of detection of the optical diffusion method, its initial concentration was increased to perform the experiments in the same time range as with the other strains. The diluted suspensions were used to inoculate 100 mL of BHI medium in an erlenmeyer. BHI medium was selected for its high-range fertility and because it was blood-free. Indeed, culture media with blood were avoided to prevent from the risk of absorbance reading errors due to their opacity. Cultures were incubated at 35 °C and at 180 rpm in a MaxQ4000 (Thermo Fisher Scientific, Waltham, MA, USA). Measurements of the optical density were performed every 30 min at 600 nm using a Cell Density meter model 40 (Thermo Fisher Scientific, Waltham, MA, USA) and disposable cuvettes. The experiments were replicated two times for each strain.

2.2. Plate preparation prior to chromatic confocal microscopy measurements

The strains were inoculated at 10^6CFU · mL^{-1} in a NaCl solution ([NaCl] = 0.45 g · L^{-1}) and then streaked on COS plates, using a PREVI® Isola instrument (bioMérieux). Agar plates were then immediately placed in an incubator at T_{incub} = 36°C ± 2°C.

2.3. Chromatic confocal microscopy experimental set-up

The principle of chromatic confocal microscopy is illustrated in Fig. 1. Early growth kinetics was followed with an Altisurf® 500 device (Altimet, Thonon-Les-Bains, France) equipped with a CL3 chromatic lens and a MG70 collimating lens (STIL SA, Aix-En-Provence, France). With this configuration of the system, the resulting vertical dynamic range was 1100 μm at a 12.7 mm working distance. Axial and lateral optical resolutions were 25 nm and 4 μm, respectively. Axial accuracy was 0.2 μm. Measurements were performed on a 2.5 mm^2 surface area per