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Characterization of Pseudomonas Aeruginosa Biofilms Grown on Different Substrates by Means of FT-IR Spectroscopy †

Marianna Portaccio *, Alessandra Fusco, Giovanna Donnarumma and Maria Lepore

Dipartimento di Medicina Sperimentale, Università della Campania "Luigi Vanvitelli", 80138 Napoli, Italy; alessandra.fusco@unicampania.it (A.F.); giovanna.donnarumma@unicampania.it (G.D.); maria.lepore@unicampania.it (M.L.)

* Correspondence: marianna.portaccio@unicampania.it.

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Abstract: Fourier Transform Infrared Spectroscopy (FT-IR) is a vibrational technique largely adopted for the study of bacterial biofilms. FT-IR is a non-destructive method allowing multiple analyses of the same biofilm. *Pseudomonas aeruginosa* represents a class of bacteria largely investigated since it is an opportunistic pathogen, and it is now considered a primary infectious agent, especially for its ability to form multi-resistant biofilms. In the present investigation, we aimed to characterize *P. aeruginosa* biofilms grown on different substrates to define better the experimental conditions more useful for investigating the interaction of these biofilms with external agents. In particular, we investigated biofilms grown on Teflon membranes, CaF_2 windows, and MirrIR slides (specific reflection FT-IR spectroscopy microscope slides). Different geometries were used for collecting spectra using the microscope stage of a Perkin Elmer Spectrum One spectrometer and a Universal Attenuated Total Reflection (UATR) device. Multiple acquisitions of spectra were done, and statistical criteria were applied for monitoring and comparing them. The positive and negative aspects of the different examined substrates for biofilm formation and acquisition modes are presented and discussed.

Keywords: FT-IR spectroscopy; *Pseudomonas aeruginosa*; bacterial biofilm; ATR; transflection

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1. Introduction

Fourier Transform Infrared spectroscopy (FT-IR) is nowadays considered a powerful tool for bacteria identification and typing [1,2]. In addition, FT-IR spectroscopy does not require specific consumable or reagent and allows also the study of the interaction between bacteria and external agents in a rapid way [1,2]. One of the advantages offered by FT-IR is the possibility to investigate the samples using different geometries and growing substrates for collecting spectra that can further simplify the measurement procedures.

In the present investigation, different spectra acquisition modes are investigating using various supports for growing bacterial biofilms. The advantages and disadvantages of the different approaches are discussed with particular attention to the measurable spectral range and the reproducibility in the spectra acquisition.

As far as concern the choice of bacterium to use for this study, we selected *Pseudomonas aeruginosa* since it represents a class of bacteria largely investigated since it is now considered to be a primary infectious agent especially for its ability to form multi-resistant biofilms [3,4].

2. Materials and Methods

2.1. Biofilm Formation

For the biofilm assay, the strain of *Pseudomonas aeruginosa* ATCC® 9027™ was grown overnight in Tryptone Soy Broth (TSB-Oxoid) at 37 °C. The overnight culture was then diluted at a concentration of 10^7 CFUs/mL, placed on sterilized CaF_2 disks, MirrIR slides and Teflon membranes and incubated overnight at 37 °C in aerobic conditions. After incubation, the biofilm formed was washed with Phosphate- Buffered-Saline (PBS, Oxoid) and fixed with 4% formalin solution.

2.2. FT-IR Measurements

The instrument that was used for the acquisition of the IR absorption spectra from *Pseudomonas aeruginosa* biofilms was a Spectrum One FTIR (PerkinElmer, Shelton, CT, USA) spectrometer, equipped with a Perkin Elmer Multiscope system infrared microscope and an MCT (mercury cadmium telluride) detector.

Microscope stage was used for the characterization of bacterial biofilms on CaF_2 disk (a transparent IR material) in transmission mode and biofilms adherent on MirrIR slides (IR reflective microscope slides produced by Kevley Technologies, Chesterland, OH, USA) in transflection mode. The background signal was acquired in a region of the support that was free of bacterial biofilm.

The spectra of *Pseudomonas aeruginosa* biofilms on Teflon membranes (TF-450, Gelman Instrument Company) were acquired in Attenuated Total Reflectance (ATR) geometry; the membrane was placed on the top of the diamond crystal of the Universal ATR (UATR) accessory of the above-mentioned FT-IR spectrometer. In this case, the background signal was acquired with the diamond crystal surface exposed to air.

The measurements were made at room temperature by collecting the signal in the spectral region between 4000 and 650 cm^{-1} using 64 scans with a spectral resolution of 4 cm^{-1} and a 5 s acquisition time for each spectrum. The biofilms were examined in different regions of about 100 × 100 μm^2 size, for biofilms on CaF_2 and MirrIR, support and three spectra were acquired for each position.

To evaluate spectra reproducibility the correlation coefficients among the spectra acquired in analogous experimental conditions were estimated.

3. Results and Discussion

The spectra of *P. aeruginosa* biofilms grown on Teflon membrane are reported in Figure 1. In the spectrum related to free Teflon membrane of Figure 1a, two peaks located at 1210, and 1150 cm^{-1} due to CF_2 stretching modes are evident and are considered a fingerprint of Teflon. In Figure 1b, the spectrum of bacterial biofilm on the membrane is reported. To eliminate the contribution of the free Teflon membrane, the difference spectrum between this spectrum and that related to the free Teflon membrane is shown in Figure 1c. In the graph the most important peaks due to biological structures are evident and in Table 1 the positions of the main contributions are reported together with their assignments.

Table 1. Spectral position of main peaks of FT-IR spectra of Figures 1c, 2 and 3. Assignments are reported according to Refs. [1,2,5].

Peak Position	Assignments	Description
3280	O-H and N-H stretching	polysaccharides and Amide A-proteins
3070	=C-H stretching	Unsaturated fatty acid
2960	C-H asym stretching	CH_3 of fatty acid chains and protein
2930	C-H asym stretching	CH_2 in fatty acid chains
2875	C-H sym stretching	CH_3 of fatty acid chains and protein

2855	C-H sym stretching	CH_2 in fatty acid chains
1640	C=O stretching C-N bending	Amide I-Protein
1540	N-H bending C-N stretching	Amide II-Protein
1450	C-H bending	CH_2 and CH_3
1400	C-O-H stretching	polysaccharides
1085	PO_2- sym stretching	nucleic acids and phospholipid
1050	C-OH stretching	polysaccharides
970	C-H bending	DNA backbone

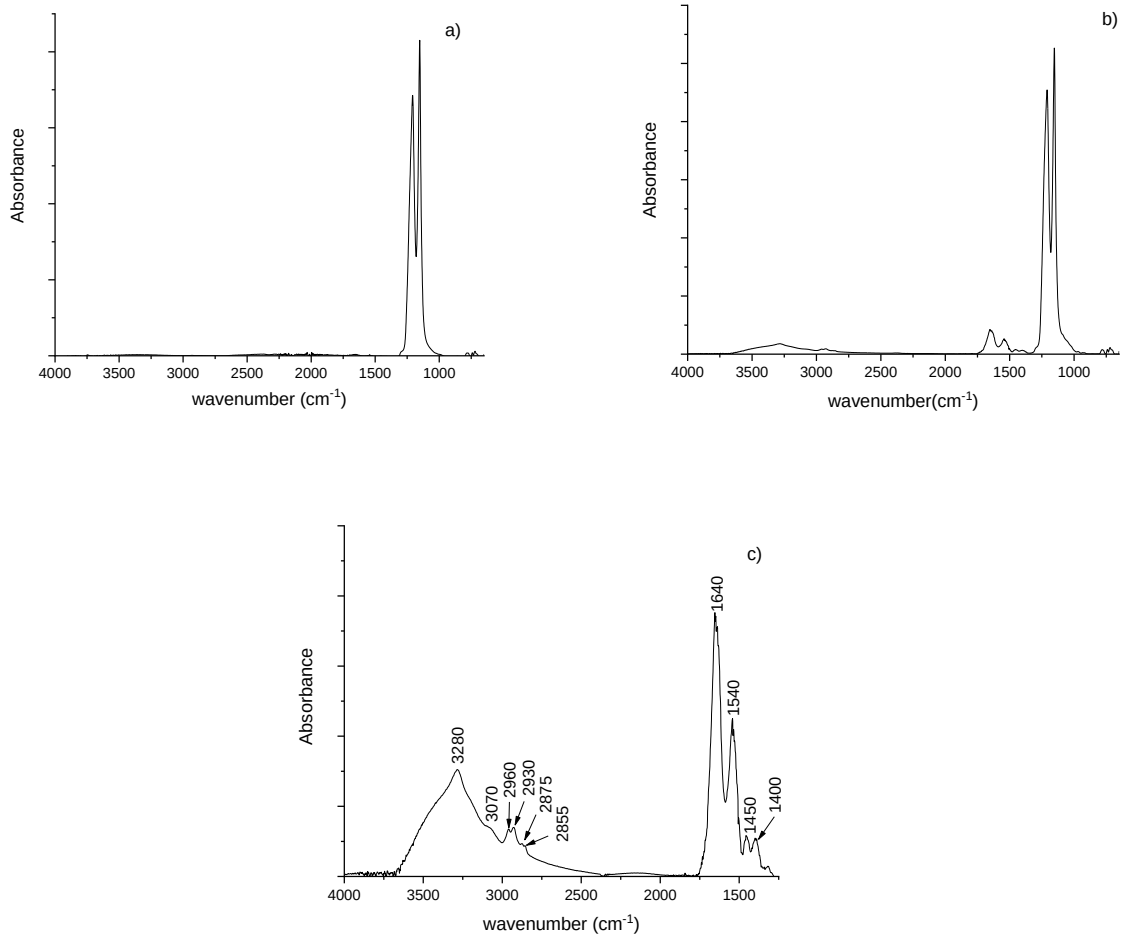

Figure 1. Average FT-IR spectrum obtained in UATR mode: (**a**) Teflon membrane; (**b**) *P. aeruginosa* biofilms on Teflon membrane; (**c**) difference spectrum of *P. aeruginosa* biofilms on Teflon membrane and Teflon membrane.

The peaks reported in Table 1 are also present in Figures 2 and 3 collected from biofilms grown on CaF_2 and MirrIR, respectively. The peaks are in similar positions with comparable intensities, also for the spectra reported in Figures 2 and 3.

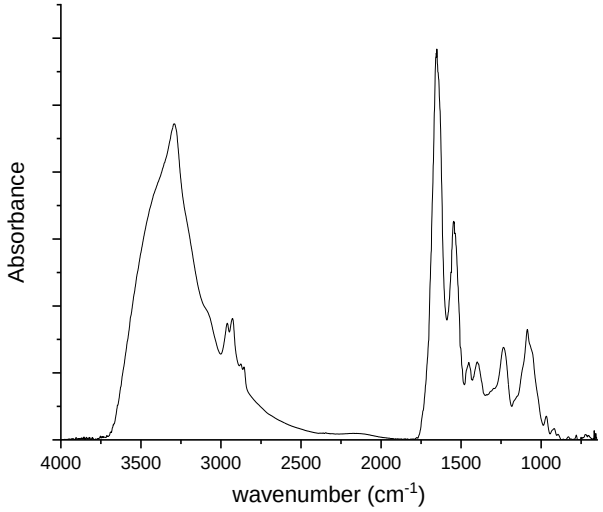

Figure 2. Average FT-IR spectrum obtained in transmittance mode for *P. aeruginosa* biofilm on CaF_2 support.

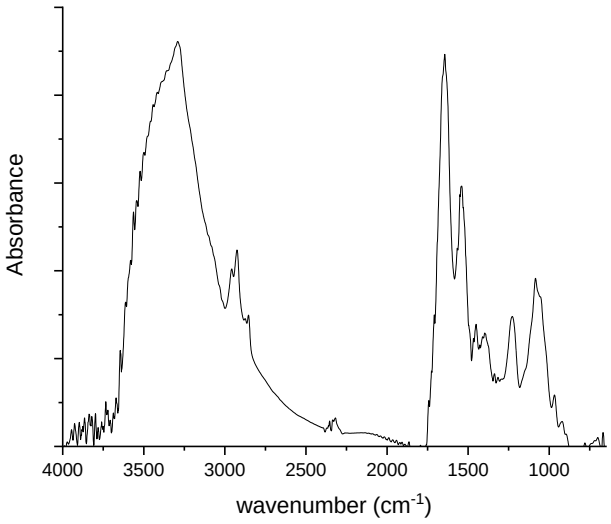

Figure 3. Average FT-IR spectrum obtained in transflection mode for *P. aeruginosa* biofilm on MirrIR support.

The different acquisition modes allow a nonidentical infrared range of the spectrum and dissimilarities in the spectra reproducibility.

In the transflection mode using MirrIR supports, it is possible to obtain significative spectra in the 4000 to 650 cm^{-1} spectral range allowing the study of contributions from all the components of bacteria (lipids, proteins, DNA, and polysaccharides) in FT-IR spectra. For UATR acquisition geometry, the presence of Teflon peaks does not permit to have valuable information for wavenumber smaller than 1300 cm^{-1}. For this reason, this approach does not permit to evaluate the contribution from DNA and polysaccharides, while the presence of lipids and proteins can be investigated without problems. For transmittance collection geometry, it is possible to study the spectral region up to 1000 cm^{-1} due to the transmittance properties of CaF_2 in infrared region. Also, for this approach, the same limitations present for UATR approach using Teflon substrates are present.

The spectra reproducibility offered by the three different acquisition modes using the above-mentioned substrates has been also investigated. The evaluation of the correlation coefficients among the various spectra acquired repeatedly in transmittance and UATR mode returned a correlation coefficient R = 0.99. In the case of tranflection acquisition, the correlation coefficient is R = 0.95. This analysis indicated that UATR geometry and transmittance are the most valuable approaches for characterizing bacterial biofilm. It is important to underline that the reproducibility of the spectra acquired is also affected by the uniformity of the bacterial film. Optical microscopy images of biofilm (not reported here) confirm that bacterial biofilms with different uniformity characteristics are obtained on the three different supports. In particular, the most uniform biofilms are obtained on CaF_2 windows and on Teflon membranes for which we have obtained the most reproducible spectra.

4. Conclusions

The above-reported considerations for the different spectral ranges available for the different supports and spectra acquisition mode together with the spectra reproducibility indicate that different choices are available in dependence on the measurement aims. For example, when the study of biofilm is focalized on bacterial proteins (important peaks in the range 1700–1500 cm^{-1}) the best choice in terms of speed of measurement and costs can be represented using UATR collection geometry with Teflon supports. Conversely, when the interest is focused on polysaccharides or nucleic acid bacterial components (range 1200–900 cm^{-1}) the use of the MirrIR support in transflection mode is suggested.

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