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Use of MALDI-TOF mass spectrometry to analyze the molecular profile of *Pseudomonas aeruginosa* biofilms grown on glass and plastic surfaces

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ABSTRACT

Biofilms are microbial sessile communities attached to surfaces that are known for causing many medical problems. A bacterial biofilm of clinical relevance is formed by the gram-negative bacteria *Pseudomonas aeruginosa*. During the formation of a biofilm, the initial adhesion of the cells is of crucial importance, and the characteristics of the contact surface have great influence on this step. In the present study, we aimed to use matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling as a new methodology to monitor *P. aeruginosa* biofilm development. Biofilms were grown within polypropylene tubes containing a glass slide, and were harvested after 3, 5, 7, 9, or 12 days of inoculation. Planktonic cells were obtained separately by centrifugation as control. Two independent MALDI-TOF experiments were performed, one by collecting biofilms from both the glass slide and the polypropylene tube internal surface, and the other by acquiring biofilms from these surfaces separately. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to evaluate the morphological progression of the biofilm. The molecular results showed that MALDI profiling is able not only to distinguish between different biofilm stages, but it is also appropriate to indicate when the biofilm cells are released at the dispersion stage, which occurred first on polypropylene surface. Finally, the present study pointed out that MALDI profiling may emerge as a promising tool for the clinical diagnostic and prognostic workup of biofilms formation and control.

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1. Introduction

Bacteria generally possess two possible distinct modes of life: the planktonic, by which the cells are able to move freely; and the sessile, by which the cells may form organized communities known as biofilms. Bacterial biofilms are commonly found in nature, being formed by colonies adhered to biotic or abiotic surfaces [1]. The adherence and growth of a bacterial biofilm involves a number of phenotypical changes, such as motility alterations, production of quorum sensing signals, and synthesis of a matrix of extracellular polymeric substances (EPS) [2].

The formation of bacterial biofilms can cause serious medical problems since they represent a reservoir of bacteria that can be shed to the body, leading to chronic infections [3]. Besides, this mode of life protects the microbial cells against antibiotics action and other physical or chemical challenges, making them difficult to eliminate with conventional therapies *in vivo* and sterilization procedures in objects [4]. The proximity of the constituent cells of a biofilm also favors the exchange of genetic materials, responsible for antibiotic resistance and consequently increases pathogen virulence and endurance [5]. Ultimately, host exacerbated responses may cause chronic inflammation and extensive tissue damage, without being able to remove the bacteria [3].

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The bacterial adhesion is one of the most important steps during biofilm formation and finally establishment of a chronic infection. This process is influenced by several factors, such as temperature, pH, bacterial concentrations, flow conditions, and surface characteristics. Among surface attributes, it is important to highlight the chemical composition, hydrophobicity, electrostatic charge, and surface roughness [3]. Whereas the biofilm undergoes several morphological (e.g. thickness and organization) and molecular changes (e.g. protein expression levels, phenotypic changes, and quorum sensing), it is interesting the use of complementary methodologies for effective understanding of the whole process. Microscopy techniques have been used to characterize the morphology and structure of bacterial biofilms [4–6]. More recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been successfully used to investigate the molecular profile of bacteria [9,10], but, from our knowledge, there is no report that use the molecular profiling data obtained by MALDI-TOF MS in order to discriminate the different stages of bacterial biofilms formation.

A biofilm that has a high impact in chronic bacterial infection, being known especially for the damage it causes in lungs of patients with cystic fibrosis, is formed by the Gram-negative bacteria *Pseudomonas aeruginosa* [7]. This is an opportunistic human pathogen that can cause acute infections in hospitalized people, especially those immunocompromised, such as patients with acquired immunodeficiency syndrome (AIDS), and neutropenic patients due to chemotherapy treatments. *P. aeruginosa* also cause deleterious infections in individuals with burns, pneumonia in patients receiving artificial ventilation, and keratitis in contact lens wearers [8,11]. The aim of the present study was to evaluate possible changes in the molecular profile of biofilms from *P. aeruginosa* in varying stages of maturity in two distinct surfaces (glass and polypropylene) using MALDI-TOF MS. In addition, the morphology of such biofilm stages was examined and compared by scanning electron microscopy (SEM) and atomic force microscopy (AFM).

2. Material and methods

2.1. Evaluation of biofilm development stages by MALDI-TOF MS

Two experiments were performed for the analyses by MALDI-TOF MS. The first experiment was performed to assess whether or not this method would be able to discriminate the stages of biofilm development as a function of the growth time. The second experiment aimed to use MALDI-TOF MS to evaluate if biofilms grown in different substrates would exhibit any detectable phenotypical distinction.

Growing planktonic cells and biofilms: Five polypropylene plastic tubes of 50 ml capacity received 20 ml each of Mueller Hinton (MH) culture medium. A rectangular microscope glass slide was placed vertically inside the tube. A pre-inoculum of *P. aeruginosa* strain ATCC 27853 was incubated in MH culture medium at 37 °C during 24 h. Ten μl of the pre-inoculum were added to the 50 ml polypropylene tubes, which were placed in a shaker with an inclination of 45° with a shaking speed of 170 rpm at 37 °C. The biofilms formed in each of the tubes were collected 3, 5, 7, 9, and 12 days after the inoculation. The culture medium of the remaining tubes was replaced in the same intervals. For the analysis of the biofilm, the tube and slide were washed two times with ultrapure water and bacteria from the tube internal surface and glass slide were collected with a sterile toothpick. Planktonic cells were collected by centrifuging 300 μl of the medium of the 5-day tube at 3000 *g* for 3 min. The supernatant was removed and the pellet was resuspended in ultrapure water and centrifuged again. This procedure was repeated and the resulting pellet was resuspended in 25 μl of ultrapure water (one μl of this suspension was used for each well of the MALDI target plate).

MALDI-TOF MS: The intact materials (biofilm and planktonic cells) were then spread in 24 wells for each sample in a polished 96-well MALDI target plate (Bruker Daltonics, Germany). One μl of α-cyano-4-hydroxycinnamic acid saturated matrix solution (10 mg/ml) was used to cover every sample and dried at room temperature prior to MALDI-TOF MS analysis. After crystallization, the samples were analyzed onto a commercial MALDI-TOF mass spectrometer MicroFlex (Bruker Daltonics, Germany) in the linear and positive mode for a range of m/z 2000–20000. The spectra were acquired automatically using a standard procedure.

MALDI Biotyper approach: The similarities among the acquired spectra of the same sample were used for generating a standardized global spectrum (MSP), so all stages of biofilm development were represented by 24 spectra, using the software MALDI Biotyper 3.0 (Bruker Daltonics). From the MSPs of samples, it was generated a dendrogram by the MALDI Biotyper method following standard procedures.

2.2. Use of MALDI-TOF MS to evaluate bacterial biofilm growth on glass and on polypropylene surfaces

Eighteen propylene tubes of 50 ml were prepared in the same way as described in the previous section. Bacteria materials of three tubes were gathered in each interval of 3, 5, 7, 9, 12, and 14 days. The culture media of the remaining tubes was replaced in the same intervals. However, in this experiment, the biological materials grown on glass and polypropylene were collected separately with a sterile toothpick, and spread over 12 wells of the MALDI-TOF target plate. Furthermore, from the tubes of the 14th day, only planktonic cells were collected in the same way as described in the previous section, and one μl of the material was spread over each of the 36 wells. All MALDI target plate wells were always covered with one μl of α-cyano-4-hydroxycinnamic acid and analyzed within 24 h.

In summary, 36 spectra were acquired in every group, and one was chosen from these as the most representative based on its common features and displayed for comparison among the experimental groups using FlexAnalysis 3.0 software (Bruker Daltonics). With the MALDI Biotyper 3.0 software, 11 MSPs were created, and subsequently clustered by a dendrogram using Euclidean distances.

2.3. Morphological characterization of the biofilms and planktonic cells

Atomic force microscopy (AFM): For the morphological characterization, polypropylene tubes of 15 ml were filled with 1 ml of MH culture medium and a circular glass coverslip was placed inside each tube. Every tube received a drop from a pre-inoculum, prepared in the same way of the previous sections, with an inoculating loop. They were placed in a shaker at the same conditions as the tubes from the previous sections. The coverslips were collected at 3, 5, 7, 9, and 12 days intervals, and every remaining tube had its medium replaced in these gaps. Two μl of planktonic cells grown for two days were spread over a coverslip and dried at room temperature. *P. aeruginosa* biofilms present in the coverslips were fixed using 1 ml of modified Karnovsky fixative (2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 0.1 M cacodylate buffer, pH 7.2) for 3 h at room temperature, being stored afterwards in cacodylate at 4 °C. Afterwards, samples were mounted on a metal sample support using double-sided adhesive tape being wrapped at the base of the instrument. The analyses were performed at ambient air with a temperature of approximately 22 °C using an atomic force microscope Shimadzu SPM-9600 (Shimadzu, Japan) equipped with

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