International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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Study and analysis of the structural-biochemical characteristics in Mollicutes biofilms formation

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International Journal of Science and Research Archive, 2024, 11(01), 057–062

Publication history: Received on 26 November 2023; revised on 04 January 2024; accepted on 07 January 2024

Article DOI: https://doi.org/10.30574/ijsra.2024.11.1.0005

Abstract

Biofilms have a wide distribution in various environments and even in devices used in medical practice, for this reason its relevance to study these structures and consider strategies to prevent their development and the effects they imply. Due to the medical importance that mycoplasmas represent, the objective of the present work was to analyze the capacity of *Mycoplasma fermentans* P140 to form a biofilm on an inert surface and to document the characteristics of the process in the formation of biofilm in Mollicutes. The interaction of the culture of *Mycoplasma fermentans* P140 with intrauterine devices for 72 hours showed by macroscopic analysis the crystal violet staining of the technique to evaluate biofilm formation, stereoscopic microscopy showed the formation of cell aggregates and by scanning electron microscopy the characteristic formation of biofilm. The search for reports referring to studies of biofilm formation in Mollicutes showed that there is recent evidence of biofilm formation in this group of microorganisms, both of veterinary medical interest and of interest in public health. Through metabolomics studies, it will be possible to demonstrate the metabolic products during the formation of biofilms in the different genera and species of these bacteria. Representing these investigations relevance to the field of public health and other ecosystems where health-disease processes are related.

Keywords: Mollicutes; Biofilms; Characteristics; Dynamic; Health-disease processes

1. Introduction

Members of the class Mollicutes are characterized by smallest self-replicating, small genomes consisting of a single circular chromosome containing 0.58 to 2.2 Mbp a low G+C content [23 to 40 mol %], the permanent lack of a cell wall and that are capable of cell-free existence. The taxonomy of this class has been extensively revised based on 16S rRNA analysis and discussed in detail elsewhere. The current taxonomic designation included in class Mollicutes comprise about 200 known species that have been detected in arthropods, plants, animals, and human [1].

It has been shown that the bacteria that persist in their hosts is due to the ability to form biofilms, since these structures exhibit different phenotypes and physiology compared to cells that are in the planktonic phase, highlighting that biofilms favor resistance against host defenses, stress and antimicrobials, in addition to facilitating the survival of *Mycoplasma putrefaciens*, *Mycoplasma cottewii*, *Mycoplasma yeatsii*, *Mycoplasma bovis* and *Mycoplasma agalactiae* in their environments, contributing to the persistence of their hosts [2].

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Host cell receptors for mycoplasmas have been documented to vary with respect to the type of host and/or mycoplasma species involved. The *Mycoplasma pneumoniae* and *Mycoplasma gallisepticum* binding sites are reported to be polypeptides that react with neuraminidase-sensitive receptors. In contrast, *Mycoplasma hominis* and *Mycoplasma salivarium* their receptors are sensitive to protease. Since 1981, it has been described that various species of mycoplasmas adhere to inert surfaces such as glass and plastic [3].

One of the advantages of living in a biofilm is that these aggregates are large and, for this reason, are not easy prey for phagocytes. The release of enzymes from the phagocytes surrounding the aggregate can cause damage to the tissues around the biofilm, and the bacteria engulfed in biofilms are resistant to antibiotics [4].

Due to the medical importance that mycoplasmas represent, the objective of the present work was to analyze the capacity of *Mycoplasma fermentans* to form a biofilm on an inert surface and to document the characteristics of the process in the formation of biofilm in Mollicutes.

2. Materials and methods

An experimental model with intrauterine devices was performed to evaluate the biofilm formation in *Mycoplasma fermentans* P140. The intrauterine devices were placed in a culture of *Mycoplasma fermentans* at a concentration of $1x10^6$ CFU/ml and incubated at 37 °C/72 hours. After incubation, the intrauterine devices were rinsed with sterile distilled water to remove non-adhered cells. They were stained with a crystal violet solution [0.5 %] for 30 minutes, followed by rinsing with sterile distilled water. Biofilm formation was analyzed by stereoscopic microscopy and scanning electron microscopy.

In addition, a search was made in PubMed for reports referring to studies of biofilm formation in Mollicutes.

3. Results

A pure culture of *Mycoplasma fermentans* P140 was obtained at a concentration of $1x10^6$CFU/ml where the intrauterine devices were immersed and interacted to facilitate interaction.

The macroscopic visual analysis of the intrauterine devices showed crystal violet staining [Figure 1A], when analyzing the samples by stereoscopic microscopy, the formation of aggregates was observed [Figure 1B]. Scanning electron microscopy showed biofilm formation by *Mycoplasma fermentans* P140 on intrauterine devices [Figure 2].

The documentary work referring to the structural-biochemical characteristics in the formation of biofilms in Mollicutes presented the following data: the analysis of biofilm growth in *Mycoplasma pneumoniae* shows traits associated with persistence and cytotoxicity, it was observed that the biofilm is robust and not very penetrable, with unusual physical properties and provides protection against immunological action. The biofilm increased resistance to antibiotics, highlighting that the resistance mechanism is essentially physical. *Mycoplasma pneumoniae* produces cytotoxic molecules, including H_2O_2, H_2S, CARDS toxins, causing vacuolation and activation of the NLRP3 inflammasome, observing that the levels of cytotoxic molecules and CARDS toxins decrease as the biofilm matures [5].

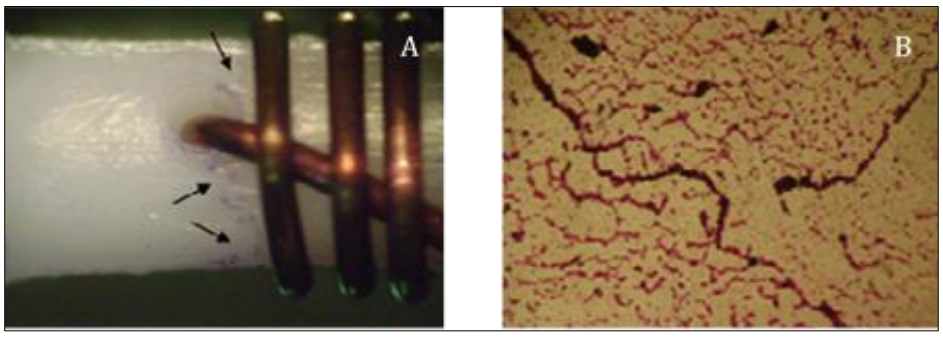

Figure 1 Macroscopic analysis of the intrauterine devices showed positive staining for crystal violet, the arrows indicate the adhesion of the dye (A), the analysis by stereoscopic microscopy (40x) showed aggregates formation (B)

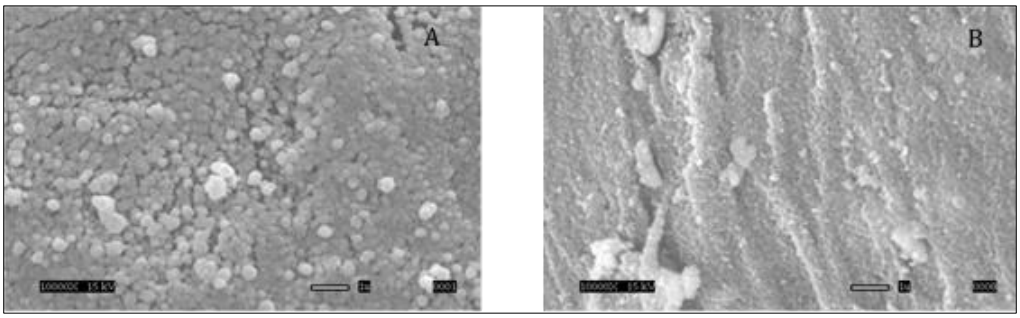

Figure 2 Scanning electron microscopy, the interaction of the intrauterine devices and *Mycoplasma fermentans* P140 culture presented cell aggregates, giving way to the biofilm (A), cohesion and the presence o**f** aggregates forming towers (B)

Several strains of *Mycoplasma anserisalpingitidis* can form a biofilm facilitating survival in the environment and providing protection against therapeutic agents. Thirty-two isolates of *Mycoplasma anserisalpingitidis* were evaluated for biofilm formation and were subjected to a temperature of 50°C [20-30 minutes], drying [16-24 hours] and different concentrations of antibiotics, 19/32 samples formed a biofilm and 13/32 were negative for biofilm formation. Biofilms showed greater resistance to heat and desiccation, compared to planktonic cultures. Biofilm formation contributes to the persistence of *Mycoplasma anserisalpingitidis* in the environments from which the isolates were obtained. Antibiotic susceptibility was not affected by biofilm formation; however, it is important to highlight that the correlation was only evaluated *in vitro* [6].

Mycoplasma hyopneumoniae forms a biofilm on cell monolayers and in lung tissue of experimentally infected pigs. It has also been shown to form a biofilm on abiotic surfaces [glass]. It is highlighted that nuclease treatment prevents the formation of biofilms on inert surfaces [glass], but does not inhibit it in porcine epithelial cells, indicating that the extracellular DNA that is located at the base of the biofilm is essential in the formation of the structure in abiotic surfaces. These data offer information on the "lifestyles" adopted by this pathogen and are related to its reduced genome and absence of wall, which allows us to study the mechanisms involved for its survival in farm environments and in pigs. Biofilm formation on glass was observed after a prolonged incubation time [10-12 days], compared to monolayer formation was rapid, with the presence of extracellular DNA playing an important role. If we forget that extracellular DNA is an essential component in the extra polymeric matrix of some microbial biofilms. Cellular variables that are a source of extracellular DNA and that initiate the biofilm formation process in Mollicutes species also play an important role [7].

Biofilms are communities of microorganisms that are encased in a polymeric matrix and grow in aggregates on biotic and abiotic surfaces. It has been seen that they can resist antimicrobials and components of the immune system *in vitro*. Some studies have shown that there is interaction between the biofilm and the host at the organ level. In mice experimentally infected with *Mycoplasma pulmonis*, a biofilm was formed at the level of the tracheal epithelium, evidenced by means of fluorescence microscopy, observing a structure and biological characteristics like biofilms formed *in vitro*. The tower structures formed in the tracheal epithelium are like the tower structures formed by mycoplasma biofilms *in vitro*, showing similar resistance to antibody penetration and dependence on a small Vsa protein. A dense packing of mycoplasma cells with a typical diameter of 500 nm. This ability of mycoplasmas to form biofilms *in vivo* may parallel the ability to form biofilms in cultured tracheal organs [8].

Mycoplasma genitalium is an important etiological agent of nongonococcal urethritis, characterized by its resistance to multidrug and chronicity, where its biofilms play an important role. It has analyzed that some bacteria capable of forming biofilms present polymeric extracellular substances composed of poly-N-acetylglucosamine, which is a crucial component of the matrix. The analysis of monosaccharides in strains of *Mycoplasma genitalium* revealed a high abundance of GlcNAc, suggesting it as a biofilm-specific extracellular polymeric substance. Chromatograms also showed abundant galactose and glucose concentrations, which are also seen in other mycoplasma species. Bacteria associated with biofilm disruption exhibit decreased viability after antibiotic treatment, compared to bacteria with intact biofilm. The data obtained suggest that the biofilm of *Mycoplasma genitalium* contributes to antibiotic resistance [9].

Mycoplasma pneumoniae forms biofilms in tissue culture models, this structure leading to chronic infection. However, it was observed that the decrease in the production of cytotoxic molecules (CARDS, H_2O_2 and H_2S toxins) is related to the possibility that these molecules are mainly produced by individual cells. This is complemented by the data that in

the phase of "biofilm towers" there is low cytotoxicity but offering physical protection. In addition, for their part, individual cells generate cytotoxicity, causing damage and with the ability to spread through the epithelium [10].

The flow method was analyzed to evaluate the biofilm growth in *Ureaplasma parvum*, favoring the removal of toxic metabolites mediated by cell death. This is the first report on the quantification of the biofilm formed by *Ureaplasma parvum*, making it possible to establish a viable biofilm that will allow the evaluation of antimicrobial agents and a better understanding of the virulence associated with adhesion [11].

Isolates of *Mycoplasma hyopneumoniae* and their association with virulence were analyzed; it was observed that the strains that showed high virulence had the capacity to form consolidated biofilms, that is, a positive correlation between the capacity to form biofilm and virulence [12].

Isolations of *Mycoplasma synoviae* were recently obtained from farms in Guangdong-China; these samples showed lower susceptibility to enrofloxacin, docycyclin, tiamulin and tylosin, compared to the type strain ATCC25204. These isolates, when evaluated against four antimicrobials, showed minimal inhibition against biofilm formation. The presence of extracellular polymeric substance (proteins, polysaccharides, extracellular DNA and lipids) was evident. This extrapolymeric substance represents the primary cement that immobilizes these microbial communities and allows subsequent resistance to antibiotics. The composition of the extracellular polymeric substance of the biofilm depends on the species that makes up the community, environmental conditions, including stress factors and nutrient availability. The intrinsic resistance of biofilm against antimicrobials is an important reason for the failure of clinical treatments, resulting in a complex barrier system. In general, microbial resistance to antibiotics can be increased by biofilm formation [13].

4. Discussion

It has been documented that 90% of microorganisms have the capacity to form biofilms and that their biosynthesis is a complex, constant and dynamic process that is characterized by four stages (adhesion, aggregation, maturation, and disintegration), involving physicochemical forces and different mechanisms. Genetic and molecular factors that regulate the biosynthesis of the extracellular matrix [14]. Due to lack of a cell wall the mycoplasmas cell membrane is exposed to the external environment, most human and mycoplasmas adhere tenaciously to the epithelial linings of the respiratory or urogenital tract; adhesion of Mollicues to host cells is a prerequisite for colonization and for infection [1].

Mycoplasma fermentans is considered a pathogen implicated in different human diseases, including rheumatoid arthritis and conditions like bacteremia. Electron microscopy studies provide high resolution to demonstrate the formation of biofilms; the analyzed images of *Mycoplasma fermentans* reveal the structure of a biofilm, including channels and with cell density in the form of towers. Different reports agree that in the biofilm there is a matrix embedded in extracellular polysaccharides (EPS), glycoproteins, glycolipids and in some cases extracellular DNA. In the present work and in comparison, with another report, it is suggested that the biofilm structures may be similar including other human mycoplasma species [15]. In addition, a work that quantified metabolites, including glycolysis compounds, amino acids, and nucleotides in the growth of human mycoplasmas, was recently reported, and the results showed that the metabolic pathways in human mycoplasmas (*Mycoplasma fermentans* and *Mycoplasma pneumoniae*) are regulated by multiple enzymatic reactions. Analyzing the metabolic pathways in planktonic cell activity and biofilm will allow a better understanding of the essential and non-essential metabolites in the formation and establishment of human mycoplasma biofilms [16,17].

Investigations have been documented that allow establishing the following question: ¿the formation of a biofilm in mycoplasmas can explain the persistence and chronicity of these microorganisms? It has not yet been conclusively explained why the Mollicutes, which are apparently fragile and lack a rigid cell wall, as they can survive in the environment, but the formation of biofilms could provide an explanation. It should be considered that biofilm formation is not simply a laboratory phenomenon but is most likely an important step in the initiation of disease in the host, which has been shown to occur *in vivo* with *Mycoplasma pulmonis*, a mouse pathogen [18].

In Mollicutes, the presence of polysaccharides EPS-I (glucose and galactose), EPS-II [GlcNAc] has been involved, which favor the formation of biofilm. In other bacteria, the polysaccharide poly-GlcNAc reported to have a critical role in biofilm formation, being like the molecule produced by *Mycoplasma pulmonis* and other Mollicutes that can form biofilm. It has been reported that there are differences between *Mycoplasma pneumoniae* strains regarding the quality of the biofilms they form, and the formation of multi-species biofilms has been reported between *Mycoplasma salivarium* and *Candida glabrata* [18-20].

Microorganisms that form biofilm develop high tolerance to antimicrobials, unlike those that grow freely. This increase is mainly associated with the inhibition of antimicrobial penetration, alteration of the microenvironment and formation of persistent microbial cells [21].

5. Conclusion

The structures of biofilms are variable and depend on several factors, including the organism, the surface, the nutrients, and the type of direction of some aqueous interfaces. The information analyzed allows us to suggest that there is still much to investigate regarding the study and to analyze the structural-biochemical characteristics in the formation of biofilms in Mollicutes, since the cells that form the biofilm are not distributed haphazardly, they are grouped in microcolonies surrounded by a matrix intermicrobial. These colonies have a microenvironment with different pH, nutrient, and oxygen concentrations, communicating with chemical signals and triggering the production of various proteins and enzymes. Thus, these studies are important in public health and in other different ecosystems that related to health-disease processes.

Compliance with ethical standards

Acknowledgments

To the institution for the facilities granted to carry out this work.

Disclosure of conflict of interest

All authors contributed equally to the conception and development of the work.

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