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Quorum Sensing molecules produced by *Pseudomonas aeruginosa* impair attachment and biofilm formation in *Candida albicans*

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ABSTRACT

Cell-to cell signaling represents maybe the most important route to control microbial behavior, virulence and infection. Although well investigated, the molecular mechanisms of Quorum Sensing (QS) communication is far of being completely understood, especially during inter-species and inter-kingdom communication. In this paper we investigated the interaction between the opportunistic pathogen *Pseudomonas aeruginosa* and *Candida albicans* empathizing on the impact of *Pseudomonas aeruginosa* main QS signaling molecules on attachment and biofilm formation of this yeast. Our results demonstrated that, among all tested molecules, N-(3-oxododecanoyl) homoserine lactone (OdDHL), 2-heptyl-4-quinolone (HHQ) and C4-HSL (N-butyryl-L-homoserine lactone) have the most significant modulatory properties regarding the attachment and biofilm formation of *C. albicans*. Moreover, the tested purified QS molecules but also *P. aeruginosa* supernatant (SN) proved to modulate *in vitro* attachment to cellular substrata in *C. albicans*.

Keywords: *Pseudomonas aeruginosa, Quorum Sensing signaling, biofilm modulation, Candida albicans, inter-kingdom signaling*.

1. INTRODUCTION

The fact that microbes are able to communicate each other and with their host is widely accepted in the scientific community. The communication process in microorganisms, named Quorum Sensing (QS) involve the production and release of small, density dependent molecules with auto-inductive properties. QS signaling may occur through bacteria belonging to the same or different species, but also between bacteria (prokaryotes) and eukaryotes, such as fungi, plant and animal cells [1,2].

Since QS signaling was proved to control numerous processes involved in virulence and pathogenicity of microbes, this process is currently considered a valuable target in the design of future efficient antimicrobial approaches. Microbial infections caused by opportunistic bacteria or yeasts are increasing and the current therapeutic approaches are becoming less effective because the resistance rates are constantly growing. It is obvious now that without an efficient and different alternative, microbial resistance rates will overwhelm our ability to create novel antibiotics and numerous infections may become untreatable [3, 4]. *Pseudomonas (P.) aeruginosa* is one of the most naturally resistant opportunistic pathogens, causing numerous difficult to treat infections. Along with an enhanced resistance gene transfer rate, the ability of this specie to produce highly organized microbial communities, named biofilms, which are tolerant to all known antimicrobials and host immune response, makes the therapy of *P. aeruginosa* infections very difficult. Key virulence factors and social behaviors, including biofilm formation are strictly controlled by QS signaling, which are represented by three main interconnected pathways in *P. aeruginosa*: Las, Rhl and Pqs. Las and Rhl QS systems are based on the production of homoserine lactones as autoinducers (AI), while in the Pqs system, AIs are represented by quinolones. The most important AIs produced by *P. aeruginosa* are the homoserine lactones: N-(3-oxododecanoyl) homoserine lactone (OdDHL) [5] and N-butanoyl-l-homoserine lactone (BHL) [6], while the most investigated quinolones are: the pseudomonas quinolone signal (PQS = 2-heptyl-3-hydroxy-4(1H)-quinolone) and its precursory molecule 2-heptyl-4-quinolone (HHQ) [7]. These molecules regulate numerous virulence related aspects in *P. aeruginosa* without necessary interfering with the fitness of the population, thus offering a new therapeutic perspective in terms of innovative antimicrobial approaches. Avoiding the use of compounds that impact on the microbial population fitness is considered to limit the selection of resistant mutants, which are frequently emerging during antibiotic therapy [8]. *P. aeruginosa* AIs may also control the interaction of the bacteria with the eukaryote host, homoserine lactones interfering with inflammatory reactions and apoptosis of the host cell [2].

Cross-kingdom signaling is more efficiently studied through model organisms and one such model is represented by the couple *P. aeruginosa – Candida (C.) albicans*. Although found normally in the human microbiota, *C. albicans* can cause major infections in immunocompromised patients. *C. albicans* is a polymorphic yeast; the switch between yeast, hyphae and pseudohyphae forms being important not only for its survival, as an adaptive response to environmental changes but also for its virulence. Besides environmental factors or mammalian serum

proteins, interaction with other species (such as *P. aeruginosa*) seem to be responsible for this form switch [9].

Interaction between *C. albicans* and *P. aeruginosa* have significant clinical implications in polymicrobial infections. These species are commonly found in mixed opportunistic infections and both of them engage in complex interactions involving their QS systems [10]. Bandara et al. (2010) investigated *in vitro* a mixt biofilm model and the results proved that *P. aeruginosa* inhibited *Candida* biofilm development, a reduce colony forming units (CFUs) of *C. albicans* being obtained [11]. According the available data, during the course of an acute infection, when high levels of AIs are secreted by *P. aeruginosa* strains, *Candida* cells present to the infection site are blocked in the yeast form. When AIs concentrations start to decrease, in a chronic infection, *C. albicans* cells could switch to filamentous form, and develop fungal biofilm [10]. Hogan et al. (2004) demonstrated that 3-oxo-C12 homoserine lactone is responsible for the inhibition of *C. albicans* filamentation in mixed infections [12]. As these two species are frequently co-isolated from severe infections is important to understand their interaction and communication in order to investigate the evolution of the infectious process and to develop new therapeutic approaches to inhibit microbial adherence and the progress of polymicrobial infections. Although, in the recent years many aspects were revealed and the interactions between these species were investigated, not all the important AIs of *P. aeruginosa* were tested. This study has proposed to investigate not only the influence of OdDHL, but also of C4-HSL, PQS and HHQ on *C. albicans* key virulence behaviors. The aim of this study was to evaluate the growth, adherence and biofilm formation during *C. albicans* and *P. aeruginosa* co-cultivation and the impact of some culture fractions and pseudomonadal purified QS molecules on the modulation of these phenotypes in *C. albicans*.

2. EXPERIMENTAL SECTION

2.1. Bacterial strains and standards.

Two laboratory microbial strains from the "Culture Collection of Microbiology Laboratory" of the Faculty of Biology, University of Bucharest, namely *Pseudomonas aeruginosa* (PAO1) and *Candida albicans* (ATCC 10231) were used in this study. The strains maintained in preservation medium (broth with 20% glycerol) at -80oC, were seeded on a solid nutrient medium (BHI, brain heart infusion agar). From microbial colonies developed on solid medium for about 20 hours, suspensions in AFS (sterile saline water) (0.5 Mc Farland for *P. aeruginosa* and 1 Mc Farland for *C. albicans*) were realized and subsequently diluted to a density of 107, 106 and 105 CFU / mL (colony forming units / mL). Alternatively, the *P. aeruginosa* colonies grown on the solid medium were incubated for 24 hours at 37 ° C to obtain the microbial culture in liquid medium represented by peptone water. The obtained cultures were subsequently centrifuged and sterilized by 0.22 µm membrane filtration in order to obtain *P. aeruginosa* cell-free cultures (supernatants -SN). QS signaling molecules produced by *P. aeruginosa* was purchased from Sigma Aldrich: OdDHL (3-oxo-C12-HSL = dodecanoyl homoserine lactone-oxo N3); C4-HSL (N-butyryl-L-homoserine lactone); HHQ (heptyl-4-quinolone); PQS (Pseudomonas quinolone signal = 3-hydroxy-4-quinolone). All compounds were diluted with HLPC grade Methanol (Sigma Aldrich) and 10 mM stock solutions were stored at −20 °C. In these experiments, 10µM represent the concentration of QS signaling molecule used.

2.2. Growth assessment (at 6h, 18h, 48h).

Assessment of *P. aeruginosa* and *C. albincans* growth after co-cultivation and assessment of *C. albicans* growth after incubation in presence of *P. aeruginosa* SN or QS molecules was carried out by determining the number of CFU /mL, using selective medium: Cetrimide agar medium was used for *P. aeruginosa* and Sabouraud Dextrose Agar medium supplemented with Chloramphenicol was used for *C. albicans*. The evaluation of growth assessment was followed in time, after different incubation time: 6h, 18h and 48h.

2.3. Evaluation of the biofilm development on inert substrata.

Biofilm development on inert substrata was assessed by the microtiter method. Overnight *C. albicans* cultures were grown in 96 multi-well plates containing Sabouraud broth in presence of *P. aeruginosa* SN or QS molecules for 24h, 48h and 72h at 37°C with shaking (shaking 200rpm). After each period of time, the plates were subsequently emptied and washed three times with phosphate buffered saline (PBS). The adherent cells were then fixed with cold methanol, stained with an alkaline 1% violet crystal solution for 15 minutes, washed with water and re-suspended in 33% acetic acid solution. The intensity of the suspension was spectrophotometrically assessed, the amount of adhered biomass being proportional to the absorbance value read at 492 nm.

2.4. Evaluation of the adherence to cellular substrata (HeLa cells).

Cell adherence assay was perform using Cravioto's adapted method. The HeLa cells monolayers were washed three times with PBS and 1 ml of fresh medium without antibiotics was added to each well. PBS suspensions of *C. albicans* obtained from mid-logarithmic phase cultures grown in Sabouraud broth were adjusted to 10^8 CFU/ml and 1 ml was used for the inoculation of each well. *P. aeruginosa* SN or QS molecules were added at a final concentration of 10µM in each well. The inoculated plates were incubated for 2 h at 37ºC. After incubation, the monolayers were washed three times with PBS, briefly fixed in cold methanol (3 min), stained with Giemsa solution (1:10) for 20 min. The plates were washed, dried at room temperature overnight and examined microscopically (×2500 magnification), to evaluate the adherence index and patterns. The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria and 100 eukaryotic cells counted on the microscopic field using an Axiolab (Zeiss) microscope. The adherence patterns were defined as: localized adherence (LA) when tight clusters of microorganisms were noticed on the HeLa

cell surface, aggregative adherence (AA) when a microbial stacked brick pattern characterize the attachment, and diffuse adherence (DA) when the bacteria adhered diffusely, covering the whole surface of the cell [25].

3. RESULTS SECTION

3.1. The evaluation of *C. albicans* growth in the presence of *P. aeruginosa.*

Our results indicate that both *P. aeruginosa* supernatants (SN) and purified QS molecules did not have any significant effect on *C. albicans* growth, the number of cells quantified by CFUs in all cases, for all investigated periods (6h, 18h, 48h) being similar. De Sordi and M¨uhlschlegel (2009) speculate that since the level of AIs in planktonic cultures varies from 10nM to 5mM and an efficient concentration, for example, for ODdHL is around 200 mM (as revealed during *in vivo* and *in vitro* tests), the molecular interaction between species is more likely to be accentuated during biofilm formation, where the level of AIs reaches around 600mM. Also, the modulation of *C. albicans* filamentation by ODdHL seems to be also dependent on the concentration of the molecule, 200 mM being sufficient for obtaining repreoducible results for this phenotype. Thus, the low concentrations from planktonic state may not be sufficient to produce any changes [13]. On the other hand, in case of *C. albicans* -*P. aeruginosa* whole culture co-cultivation, a decreased number of CFUs was observed at 18h and 48h (Figure 1). These results suggest that soluble mediators are not sufficient for the modulation of *C. albicans* growth, direct contact of the cells being probably necessary. However, the fact that the utilized QS molecules do not interfere with the microbial population fitness (but may modulate virulence behaviors) may recommend them as a strategy to control infections by modulating the virulence of the pathogen (especially when opportunistic microbes are involved), without necessary killing the cells.

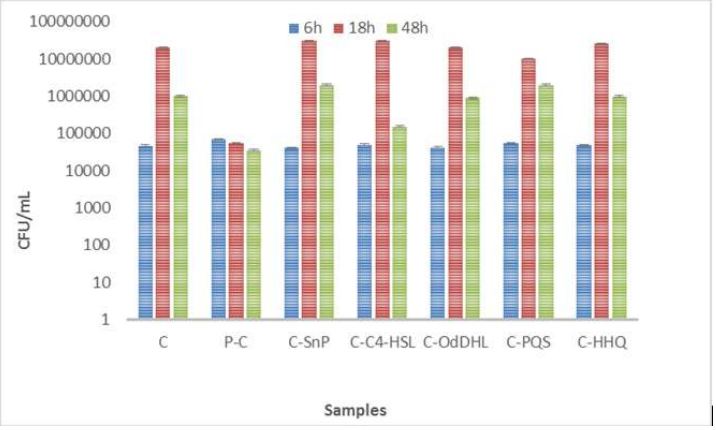

Figure 1. Graphic representation of CFUs after incubation of *C. albicans* (C.) in presence of *P. aeruginosa* culture (P.), supernatant (Sn) and purified signaling molecules.

2.2. Influence of *P. aeruginosa* on the biofilm development to inert substrata

However, adherence and biofilm development may be significantly modulated by both *P. aeruginosa* SN and purified QS molecules. The results correspond to the literature data according to which a series of specialized processes including formation of biofilms, virulence and attachment are adjusted via this molecular communication system in bacteria, but also during inter-kingdom signaling. All the fractions used in this study decreased *C. albicans* capacity to adhere to the inert substrata, this comportment being maintained throughout the entire experiment (samples were harvested at 24h, 48h and 72h). From all tested molecules, OdDHL and HHQ were most effective against biofilm formation, this phenotype being significantly reduced in their presence. In the case of co-cultivation of *P. aeruginosa* and *C. albicans* culture, the real value of *Candida* biofilm couldn't be read, the high absorbance observed being also due to the development of *Pseudomonas aeruginosa* cells (Figure 2).

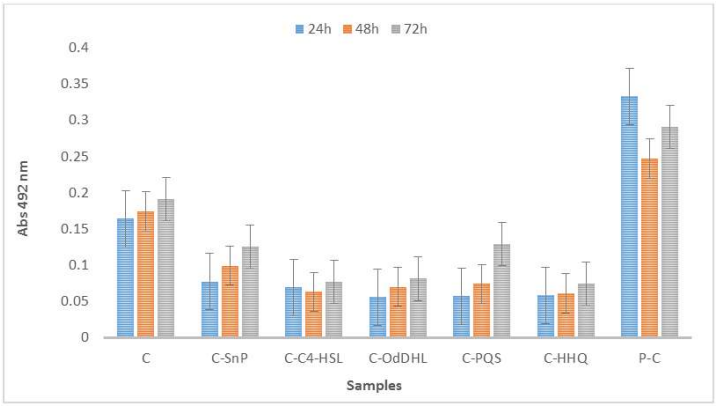

Figure 2. The comparative level of the attachment and biofilm formation ability of *C. albicans* ATCC strain to the inert substrata in presence of *P. aeruginosa* culture (P.), supernatant (Sn) and purified signaling molecules, quantified by measuring the absorbance at 492 nm.

3.3. Influence of *P. aeruginosa* on the *C. albicans* adherence to cellular substrata.

The capacity of attachment to a surface was also observed on cellular substrata, *C. albicans* tested strain being able to adhere to HeLa cells, although with a reduce rate (10% adherence index). In presence of signaling molecule, the percentage of adherence proved to increase around 35 to 50%. The most predominant adherence pattern was aggregative, and diffuse- aggregative (Table 1 and Figure 3).

Table 1. The adherence index and *patterns* of *C. albicans* ATCC strain to cellular substrata in presence of *P. aeruginosa* culture (P.), supernatant (Sn) and purified signaling molecules.

Sample	Adherence pattern	Adherence index (%)
C	diffuse - aggregative	10
C-P	diffuse - aggregative	5
C-SnP	localized - aggregative	10
C-C4-HSL	aggregative	45
C-OdDHL	aggregative	40
C-PQS	aggregative	50
C-HHQ	diffuse - aggregative	35

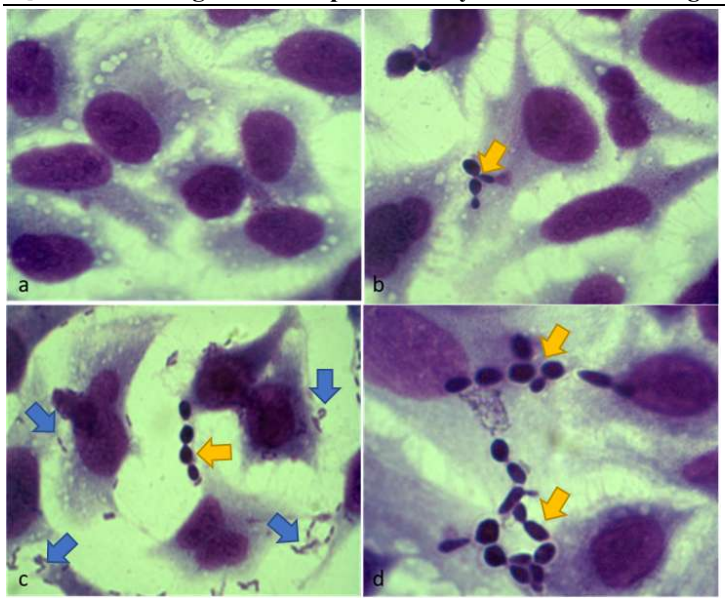

Figure 3. Main aspects observed for the adherence of *C. albicans* to HeLa cells: a- control HeLa cells, b- control- untreated *C. albicans* , c- *C. albicans* + *P aeruginosa*, d- *C. albicans* + PQS (Giemsa staining, 1000X). *C. albicans* cells are pointed with yellow arrows, while *P. aeruginosa* cells are highlighted by using blue arrows.

The interactions mediated by QS molecules occurring between pathogens from different species during the infectious process are far from being fully decrypted. Even if in the last years many researchers are interested and focus on these aspects, QS remains the best characterized communication and signaling method within a bacterial species, but competitive or cooperative signaling processes that take place between groups of different species or even different kingdoms is still poorly investigated. *C. albicans* and *P. aeruginosa* are often implicated in infections with polymicrobial biofilms and thus are frequently involved in nosocomial infections, or infection in immunocompromised individuals. Taking into account that these two microorganisms are often co-isolated from the same infectious processes, for example the sputum patients with cystic fibrosis (CF), our research investigate some aspects that conduct their interaction, as growth, adherence and the capacity to form biofilms. Our results revealed that indeed *P. aeruginosa* and *C. albicans* interfere during cell-to-cell contact but their behavior and virulence may be also modulated through QS signaling molecules (cell-to-cell contact being unnecessary). Moreover, *Pseudomonas aeruginosa* inhibit *C. albicans* growth, results reported also by other *in vivo* and *in vitro* studies [10]. Brand et al. (2008) showed also that *P. aeruginosa* had the capacity to kill *C. albicans* hyphal cells, but not *C. albicans* yeast cells, effects attributed to piocianin, a toxin molecules produced by this opportunistic pathogen, which alters the cell wall of *C. albicans* [14]. Regarding QS molecules, it seems that they do not modulate the growth of *Candida* yeast cells and basically do not significantly interfere with the population fitness, which determined us to investigate other aspects in which these mediators could be involved. In literature, are described many important processes in which communication through QS mechanism is essential, as the production of antibiotics, bioluminescence, adherence and biofilms development, competence, sporulation, motility and virulence [15-17]. The adherence capacity to the inert substrate is a major problem for medical field, because bacteria can adhere and form biofilms on the inert surfaces, and thus the risk of nosocomial infectious is increased. In some cases, biofilms are populated by a single species, while in others biofilms involve a diverse range of microbial species. For residents of a biofilm, a community development offers significant advantages. For example, bacteria living in biofilms are significantly more tolerant to antibiotics and antibacterial substances and receive shelter from environmental stress factors, including host immune system attack. Furthermore, increased cell proximity facilitates horizontal gene transfer and sharing metabolic products between community members [18]. Taking into account all these aspects of the problem, and considering the possible role of *Pseudomonas* QS molecules in biofilms development, the study of the adhesion capacity of the bacterial strains to inert and cellular substrata became evident the next steps we have followed. Indeed, our research demonstrated that, when high concentration of these mediators are accumulated in *P. aeruginosa* cell free SN as well when purified QS molecules were added in a *C. albicans* culture, its adherence and capacity to develop biofilm is affected. Similar results were observed by McAlester et al. (2008) in a study on OdDHL, when these molecules inhibit the capacity of *C. albicans* to switch from the yeast to hyphal form. This morphological switch that occur when *C. albicans* senses the presence of *P. aeruginosa* (by QS molecules) represent for fungus a survival strategy, but there is well known that in the filamentous form, *Candida* is more virulent and with has a higher capacity to adhere to a substratum, so is clear that by inhibiting this switch, *Candida* adherence is in fact inhibited [19]. Reen et al. (2011) [20] obtained similar results using PQS, and its immediate precursor, 2-heptyl-4-quinolone (HHQ). Our results confirm these data, but for all investigated molecules, the highest capacity to repress *C. albicans* biofilm formation being observed in case of OdDHL and HHQ [21, 22].

When we tested attachment to a cellular substrate, the addition of *P. aeruginosa* signaling molecules caused the changes in *Candida* adherence *pattern* to aggregative one, formation of cell aggregate, with budding cells and even some small filament being observed. These results show that these signaling molecules are not important only for intra-species communication, but are implicated also in the interaction with other organisms, belonging to other species and even other kingdoms such as *C. albicans*. This interaction is not unilateral; *Candida* is able also to produce some molecules (such as farnesol) as chemical signals [23-27], so more studies are necessary for elucidate these aspects in order to better understand the interaction between these two species during infectious process and to choose the best therapeutic approach [27].

4. CONCLUSIONS

To conclude, molecular signaling represents an important aspect for the virulence and pathogenicity of microorganisms and they may be considered key elements in the progress of the infectious process and maybe in the success of therapy. Moreover, the modulation of molecular communication itself could represent an efficient target for future antimicrobial strategies, based on the modulation of key behaviors and virulence without necessary interfering with the fitness of the microbial population, which may

reduce the rate of emergence of resistant microbial cells. This study supports the idea that *P. aeruginosa* main QS signaling molecules may specifically modulate key virulence behaviors in *C. albicans* such as attachment and biofilm formation, without necessary interfering with the fitness of microbial population, which may represent an alternative solution in the control of the progression of the infectious process and thus the severity of infection.

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