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Effect of intraspecies interactions on *Pseudomonas aeruginosa* biofilm formation and gene expression

Mariana Alcântara Calomino[1], Michelle Pereira Silva[1], Heidi Pauer[2], Beatriz Guitton Renaud Baptista de Oliveira[3], Francislene Juliana Martins[1], Gabriel Luis Cavalcanti Valente[1*], Geraldo Renato de Paula[1] and Felipe Lopes Teixeira[1,2]

[1]Programa de Pós-Graduação em Ciências Aplicadas a Produtos para Saúde, Universidade Federal Fluminense, Rua Miguel de Frias, 9, 24220-900, Niterói, Rio de Janeiro, Brazil. [2]The University of Kansas, Lawrence, Kansas, USA. [3]Programa de Pós-Graduação em Ciências do Cuidado em Saúde, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. *Author for correspondence. E-mail: fgabrielvalente@gmail.com

ABSTRACT. *Pseudomonas aeruginosa* is a common pathogen of acute and chronic infections that can increase risk to burned patients and those with chronic wounds, especially when forming biofilms. These complex ecosystems are formed by several bacteria that communicate, cooperate, and compete with each other. To enhance its survival capacity in such a competitive environment, *P. aeruginosa* produces secondary metabolites with a range of biological activities, among other factors. Here, we evaluated how such bioactive molecules mediate intraspecies interactions to modulate *P. aeruginosa* biofilm formation. An overall decrease in biofilm formation on the surface of stainless steel coupons was observed for both dual-strain and single-strain biofilms formed in the presence of secondary metabolites extracted from other *P. aeruginosa* strains. Modulation of biofilm formation by these metabolites during *P. aeruginosa* intraspecies interactions suggests a role for these molecules in strain competition. The most likely mechanism by which it occurs might be modulation of iron uptake. Among the genes analyzed, those related to import (*tonB1*) and biosynthesis (*pvdS*) of the siderophore pyoverdine were the most downregulated during exposure to the metabolite extracts. Exploring *P. aeruginosa* intraspecies interactions during biofilm formation might be an interesting approach to understand its lifestyle better and, thus, the mechanisms involved in the species' biofilm formation.

Keywords: Iron uptake modulation; production of biofilms; quorum sensing; pyoverdine; secondary metabolites.

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Introduction

Pseudomonas aeruginosa is a Gram-negative aerobic bacterium frequently found in the environment and one of the most common pathogens responsible for a multitude of acute and chronic infections, including burn wound infections, human skin wounds, and bloodstream infections (Moradali, Ghods, & Rehm, 2017; Jurado-Martín, Sainz-Mejías, & McClean, 2021; Reynolds & Kollef, 2021). *P. aeruginosa* is one of the main Gram-negative bacteria involved in healthcare-associated infections (Weiner-Lastinger et al., 2020). Furthermore, in this era of antimicrobial resistance crisis, carbapenem-resistant *P. aeruginosa* is, according to the World Health Organization, a critical priority pathogen that needs new antibiotics to treat its infections (Tacconelli et al., 2018). It is believed that the ability of *P. aeruginosa* to cause disease is mainly due to its ability to persist in two phenotypic states, a free-living (planktonic) state and a sessile state, in which there is an aggregate of bacterial cells, the biofilm (Percival, McCarty, & Lipsky, 2015). The latter is a problem in patient care, as it contributes to its ability to cause recalcitrant infections (Lebeaux, Ghigo, & Beloin, 2014).

Quorum sensing (QS) is one of the major regulatory systems controlling biofilm formation in *P. aeruginosa*. QS is an intercellular communication mechanism bacteria use to coordinate their behavior in response to changes in population density. Small signaling molecules, called autoinducers, are detected by cognate cellular receptors that, once bound to an autoinducer, activate transcription of QS target genes (Lee & Zhang, 2015; Lee & Yoon, 2017). In *P. aeruginosa*, the expression of several hundred genes is controlled by at least four different QS systems (Las, Rhl, PQS, and IQS), each producing and sensing a different autoinducer (Wagner, Gillis, & Iglewski, 2004; Lee & Zhang, 2015).

Several environmental signals can induce the breakdown of bacterial biofilms, such as oxygen supply, nitric oxide, pH, and some chemicals (Sauer et al., 2004; Schleheck et al., 2009; An, Wu, & Zhang, 2010).

Availability of iron can also contribute to dispersing formed biofilms (Gjermansen, Ragas, Sternberg, Molin, & Tolker-Nielsen, 2005). To mitigate the effects of iron deprivation, *P. aeruginosa* produces siderophores such as pyoverdine (Schalk & Guillon, 2013), which binds to extracellular iron to help transport it into the cells. Pyoverdine is not only essential for normal biofilm formation under low iron conditions (Banin, Vasil, & Greenberg, 2005), but it also confers a competitive advantage to producing strains (Dell'Anno et al., 2022).

A large number of microorganisms are present in skin wounds. Contamination and infection increase the risk to burned patients and those with chronic wounds, especially in a biofilm context, where several species communicate, cooperate, and compete with each other.

Pseudomonas aeruginosa versatile metabolism, which allows it to thrive in numerous environments, including wounds, might lead to the production of a range of secondary metabolites - bioactive small molecules that contribute to bacteria competitiveness but are not required for survival (Craney, Ahmed, & Nodwell, 2013). Biological active small molecules can modulate QS and interfere with *P. aeruginosa* pathogenic behavior (Mattmann & Blackwell, 2010) such as biofilm formation or pyoverdine production. Thus, the present study aimed to evaluate if strain-specific secondary metabolites would affect the formation of single-strain *P. aeruginosa* biofilms, and to analyze how these bioactive small molecules influence the expression of genes associated with QS systems and pyoverdine import and biosynthesis.

Materials and methods

Bacterial strains and study design

Pseudomonas aeruginosa strains used in this study were obtained from the culture collection of the Laboratory of Microbiological Control of the School of Pharmacy at *Universidade Federal Fluminense*. They were originally isolated from burn wounds, chronic venous ulcers, and bloodstream infections. The reference strains ATCC 27853 and PAO1 were also used. *P. aeruginosa* strains were grown in Tryptic Soy Broth (TSB) or Agar (TSA) at 35ºC (±2ºC).

Interactions between selected strains were analyzed either in co-culture with two strains or monocultures supplemented with metabolites extracted from the other strain. Interactions with the following strains were carried out: ATCC 27853 (Reference strain) with 390 (Blood culture strain) or K5 (Burn wound strain); PAO1 (Reference strain) with K4 (Burn wound) or 23 (Blood culture strain); and F1 (Chronic ulcer from diabetic patient strain) with 244 (Blood culture strain). Strain interactions were established based on resistance to ciprofloxacin, which was used as a phenotypic marker to distinguish between strains in co-culture experiments. *P. aeruginosa* strains were tested for ciprofloxacin resistance according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2018), and the concentration of 4 μg mL^{-1} of ciprofloxacin was used for strain selection in co-culture experiments.

Extraction of metabolites produced by *P. aeruginosa*

The extraction of metabolites was performed as previously described (Antunes et al., 2014), with some modifications. Strains were inoculated in TSA and incubated at 35ºC (± 2 ºC) overnight. Colonies were then transferred to tubes containing 5.0 mL of TSB and incubated overnight at 35ºC (± 2 ºC). Afterward, the bacterial culture was mixed with ethyl acetate in a 1:1 (v v^{-1}) ratio. The mixture was transferred to a separatory funnel, where the phases were separated. The organic phase was collected and dried on rotavapor. The dry extract was resuspended in sterile water to the original concentration and stored at -20 ºC until used.

Biofilm formation test on stainless steel coupon

Biofilm formation on the surface of stainless steel AISI 304 #4 coupons (roughness 0.36 μm; size 10 x 10 x 1 mm) was analyzed as previously described (Casarin et al., 2016). *P. aeruginosa* strains were inoculated in TSA and incubated overnight at 35 °C (± 2 °C). Colonies were transferred to 5.0 mL of 0.9% saline solution until reaching turbidity equivalent to 0.5 on the McFarland scale. The standardized suspension was diluted in 0.9% saline to obtain a 10^4 CFU mL^{-1} concentration. Wells of a 24-well culture plate (Kasvi®) were filled separately with 900 μL of TSB and 100 μL of a monoculture bacterial suspension; 200 μL of co-culture (100 μL of each strain suspension) and 1800 μL of TSB; or 100 μL of a monoculture bacterial suspension, 100 μL of extracted metabolites, and 800 μL of TSB. Sterile coupons were placed in the wells containing the suspensions and the plates were incubated overnight at 35 ºC (± 2 ºC).

Adhered cells were quantified according to Parizzi, Andrade, Silva, Soares, and Silva (2004). The stainless steel coupons were removed from the plate with the aid of a sterile forceps and immersed in a tube (Falcon®) containing 10.0 mL of 0.9% saline solution for 1 min. to remove planktonic cells. Then they were removed from this solution, immersed in tubes (Falcon®) containing 5.0 mL of 0.9% saline solution, and vortexed (QL-901 Vortex - Vertex; Brazil) for 2 min. to detach the sessile cells. The resulting suspension was serially diluted in 0.9% saline solution and 50 µL of each dilution were inoculated in TSA with or without ciprofloxacin (4 µg mL^{-1}) and incubated at 35 °C (± 2 °C) overnight CFU counting. The entire experiment was performed in triplicate.

Analysis of gene expression by quantitative PCR

Alterations in gene expression caused by *P. aeruginosa* secondary metabolites were analyzed by quantitative PCR (qPCR). Six genes associated with *P. aeruginosa* QS, either regulators of QS systems (*lasR, pqsR, rhlR*) or genes regulated by them (*lasB, pqsA,* and *rhlA*), and two genes associated with import (*tonB1*) and biosynthesis (*pvdS*) of pyoverdine were selected for the analysis. Primers used to amplify each gene are listed in Table 1.

TSB cultures of *P. aeruginosa* were grown overnight at 35 ºC (± 2 ºC) with or without the addition of metabolite extracts at 10% final concentration (v v^{-1}). After incubation, total RNA from cultures in the logarithmic growth phase (OD_{600} = 0.2 to 0.4) was extracted using the PureLink™ RNA Mini Kit (Ambion), according to the manufacturer's instructions. Residual DNA was removed after treatment with RQ1 RNase-Free DNase (Promega). Reverse transcription of 500 ng of mRNA into cDNA was performed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's protocol. qPCR was carried out according to the manufacturer's instructions. Each qPCR reaction was performed in a standard mixture with a final volume of 12 µL containing 6 µL of GoTaq® qPCR Master Mix (Promega), 0.3 µL of each 10 µM primer, 0.2 µL of CXR dye, 3.2 µL of Nuclease-Free water and 2 µL of cDNA. Sample reactions were amplified using StepOnePlus™ Real-Time PCR System (Applied Biosystems). The *rpoD* gene, which encodes the sigma factor 70 in *P. aeruginosa* (Livak & Schmittgen, 2001), was used as endogenous control. The relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). All tests were performed in duplicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.2.1). Wilcoxon matched pairs nonparametric test was used to evaluate differences in relative sessile cell count between co-cultures and monocultures supplemented with metabolites. Two-Way ANOVA with corrected Turkey's post-hoc test was used to analyze the differences in relative expression between genes. Statistical significance was determined by a p-value < 0.05.

Table 1. Primers used by quantitative PCR.

Gene	Primer name	Sequence 5'- 3'
rpoD	RpoD -F	GGGCTGTCTCGAATACGTTGA
	RpoD- R	ACCTGCCGGAGGATATTTCC
pqsR	PqsR -F	GAAAACTTCGACGACATGCTG
	PqsR -R	CGTAGAGTTCGCTGAGGACTG
pqsA	PqsA -F	GCCTGTTTCCTGCAATACACC
	PqsA -R	AATTCGAATACAGCCGGTCTCC
rhlR	RhlR -F	ACAATTTGCTCAGCGTGCTTT
	RhlR -R	CTTCTGGGTCAGCAACTCGAT
rhlA	RhlA -F	CGAACATTTCAACGTGGTGCT
	RhlA -R	GATTCCACCTCGTCGTCCTT
lasR	LasR -F	TAAGGACAGCCAGGACTACGA
	LasR -R	GCTCTGGGTACAGTGACTGAC
tonB1	TonB1-F	GAGGAAGTGCTGATCCCCTA
	TonB1-R	AAATTCACCTGCATGGTCTTC
pvdS	PvdS -F	CAGATCACTTCGTCGTTCAAG
	PvdS -R	GTAGTTGATGTGCGAGGTTTC
lasB	LasB -F	GGAATGAACGAAGCGTTCTC
	LasB- R	GGTCCAGTAGTAGCGGTTGG

Results

Interaction between *P. aeruginosa* strains in stainless steel coupon biofilms

Interaction between strains during biofilm formation in stainless steel coupon revealed an overall reduction in sessile cell count in most co-cultures. Co-cultured strains count ranged from -6.84 to 13.38-fold (median: -1.78) their respective cell counts in monoculture. Only strains K5 grown in the presence of ATCC 27853 and PAO1 grown in the presence of K4 had an increase in CFU count compared to their respective monoculture. All other strains had decreased sessile cell counts in the co-cultured biofilms (Figure 1).

A similar scenario was observed in monocultures supplemented with metabolites extracted from respective co-culture strains. Cell counts ranged from -5.52- to 2.86-fold (median: -1.48) the count of non-supplemented monocultures. Although the reduction in sessile cell count median was smaller in monocultures grown in the presence of metabolites compared to co-cultures, no statistical difference was observed between the two groups (Figure 1).

Modulation of *P. aeruginosa* gene expression by secondary metabolites

Analysis of qPCR revealed that metabolites produced by different strains of *P. aeruginosa* can modulate the expression of QS-related genes in the species (Figure 2A). *pqsR* expression ranged from -13.09- to 2.73-fold (median: -1.11); *pqsA* ranged from -14.05- to 2.69-fold (median: 0.01); *lasR* from -3.22- to 14.59-fold (median: -1.12); *lasB* from -14.79- to 5.12-fold (median: -4.72); *rhlR* from -3.4- to 4.78-fold (median: 0.03); and *rhlA* from -13.26- to 2.8-fold (median: 1.05). Differential expression was observed for all genes, with both up- and downregulation detected. However, the median relative expression for most genes was similar to the expression levels found in the control groups not exposed to the metabolites, showing that these chemical interactions between *P. aeruginosa* strains, though able to modulate QS, are diverse and might balance each other out *in vivo*. An exception was observed for *lasB*, which was the only gene that had an overall reduction in expression, with an average of approximately 5-fold downregulation among tested strains. Increased expression of *lasB* was correlated with increased expression of *lasR*: The three strains with upregulated *lasB* had the highest expressions of *lasR*.

Modulation of pyoverdine import and biosynthesis gene expression by *P. aeruginosa* metabolites was more evident than what we observed for QS genes (Figure 2B). *tonB1* expression ranged from -63.67- to 2.23-fold (median: -1.79); and *pvdS* ranged from -68.23- to 3.48-fold (median: -1.84). In addition to lower medians overall, the average expression (-8.83 and -8.80-fold, respectively) was also much lower, influenced by more extreme downregulation values over 60-fold reduction for both genes.

Discussion

The success of antibiotic-resistant pathogens such as *P. aeruginosa* depends on their ability to actively adapt. Among many factors, the metabolic flexibility contained in the genome of *P. aeruginosa* allows this pathogen to adapt to a competitive environment, providing growth in different physiological conditions (Rojo, 2010; Marques & Nelson, 2019; Riquelme et al., 2020). Given its versatile metabolism, diverse pools of secondary metabolites could be produced by different *P. aeruginosa* strains, which might influence intraspecies cell signaling. Thus, we analyzed if chemical interactions between different strains mediated by such metabolites could affect biofilm formation by *P. aeruginosa*. These metabolite-mediated intraspecies interactions led to an overall decrease in biofilm formation and reduced the expression of genes related to biofilm formation, especially those associated with iron acquisition. Modulation of biofilm formation and other virulence traits by small molecules have been previously reported (Antunes et al., 2014; Glatthardt et al., 2020; Pauer et al., 2021), but here we show that metabolites contribute to biofilm modulation during *P. aeruginosa* intraspecies interactions with a decrease in biofilm formation, suggesting a role of these molecules in strain competition.

Although we observed decreased biofilm formation in stainless steel coupons in both metabolite-supplemented cultures and co-cultures, the behavior of individual strains diverged slightly between the two conditions. Rieusset et al. (2020) showed that the diversity of metabolites produced by some *Pseudomonas* species can vary significantly between sessile or planktonic lifestyles. Therefore, since the metabolites used here were extracted from planktonic monocultures, they probably diverged in composition compared to the metabolite pool produced by the co-cultures during biofilm formation, hence the slight difference between the two conditions.

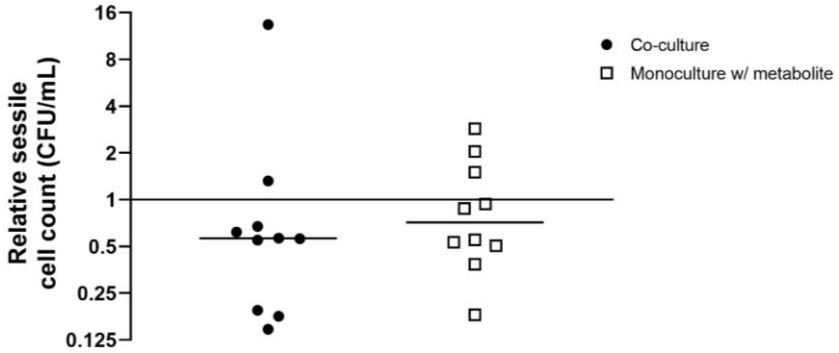

Figure 1. Interaction between *Pseudomonas aeruginosa* strains in stainless steel coupon biofilms. Sessile cell counts of *P. aeruginosa* strains attached to stainless steel coupons after growth in co-culture or monoculture in the presence of metabolites extracted from *P. aeruginosa* strains. Adhered cells from monocultures grown without metabolites were used as a reference for the relative cell count.

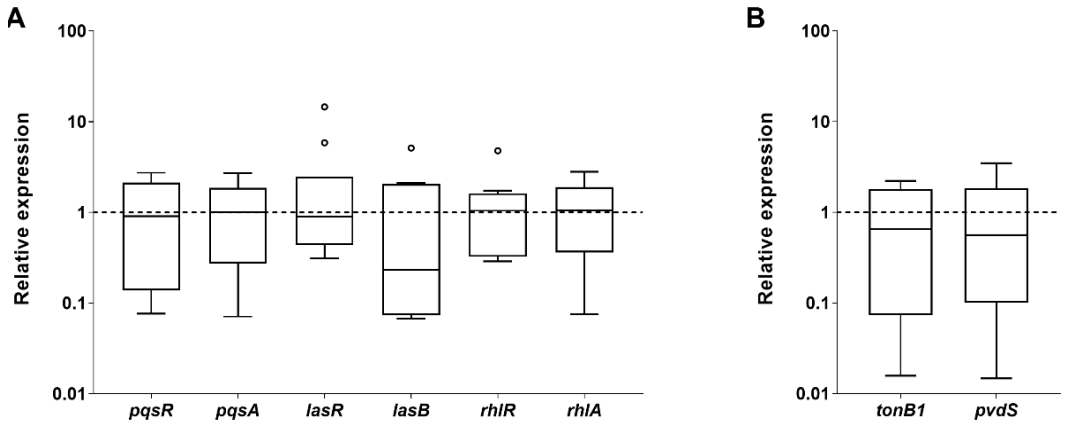

Figure 2. Modulation of *Pseudomonas aeruginosa* gene expression by secondary metabolites**.** Differential expression of genes associated with (A) quorum sensing and (B) pyoverdine import and biosynthesis caused by growth in the presence of metabolite extracts. RNA from monocultures of *P. aeruginosa* strains grown in the presence or absence of metabolites was extracted and converted into cDNA, which was used for qPCR analysis. The relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method. Tukey method was used to determine outliers, represented by individual points in the graph.

Quorum sensing plays a major role in biofilm formation and *P. aeruginosa* QS network consists of several interconnected signaling pathways (Lee & Zhang, 2015). The QS transcriptional regulator PqsR regulates critical virulence factors for acute, persistent, and recurrent *P. aeruginosa* infections (Cao et al., 2001; Déziel et al., 2005). PqsR also triggers the expression of the PqsA enzyme. Both PqsR and PqsA have been associated with the formation of biofilms (Müsken, Di Fiore, Dötsch, Fischer, & Häussler, 2010; Bala, Kumar, Chhibber, & Harjai, 2015), but the reduced biofilm formation in the presence of *P. aeruginosa* metabolites could not be linked to differential expression of this QS pathway in our work.

The QS regulator RhlR did not seem to have an impact on the decreased biofilm formation either. The RhlR regulator is closely linked with biofilm formation (Mukherjee, Moustafa, Smith, Goldberg, & Bassler, 2017). The expression of *rhlA*, one of the genes in RhlR regulon, is the only requirement for the initiation of rhamnolipid synthesis in *P. aeruginosa*. These biosurfactants produced via RhlA product proved to be important for the maintenance of water channels between multicellular structures in biofilms, but if added to bacterial cultures at the time of inoculation, they provided a complete block of biofilm formation (Davey, Caiazza, & O'Toole, 2003).

The Las system is at the top of the *P. aeruginosa* QS signaling hierarchy, and LasR acts as a master regulator that activates the expression of Rhl and PQS systems (Juhas, Eberl, & Tümmler, 2005; Cabeen, 2014). LasR positively regulates biofilm formation (Elnegery, Mowafy, Zahra, & El-Khier, 2021), but we found little evidence in our study that the decreased count of sessile cells in the presence of *P. aeruginosa* metabolites was due to alteration in *lasR* expression.

Even though we observed a variation in the expression of QS-related genes between strains in the presence of a diverse pool of *P. aeruginosa* metabolites, no difference to untreated control could be seen when the strains were grouped in a single batch. *P. aeruginosa* has a very robust pangenome, with estimates of 10,000 to 40,000 genes distributed among the different strains of the species (Mielko et al., 2019), and an extensive

QS network, with at least four interconnected pathways (Lee & Zhang, 2015). These complex features make it difficult to interpret data of isolated QS when comparing diverse strains and, thus, more studies are needed to understand the impact of the interactions between *P. aeruginosa* strains on QS systems.

However, one gene regulated by at least two of the major *P. aeruginosa* QS pathways, *lasB*, had a more consistent deregulation when compared to the other genes. The elastase LasB is an important virulence factor secreted by *P. aeruginosa* and the full activation of its promoter is dependent on both LasR and RhlR (Lee & Zhang, 2015). LasB has a role in extracellular iron acquisition by cleavage of iron-bound host proteins (Wolz et al., 1994). It has been previously reported that LasB is necessary so pyoverdine can acquire iron from human transferrin (Döring, Pfestorf, Botzenhart, & Abdallah, 1988).

Previous studies have shown that siderophores, including pyoverdine, play an important role in biofilm formation (Banin et al., 2005; Ojha & Hatfull, 2007). In our study, we observed downregulation of genes related to pyoverdine import and biosynthesis, suggesting that the decreased biofilm formation observed in monocultures supplemented with metabolite extracts might be related to deregulation of enzymes related to iron metabolism. Iron availability serves as a signal in biofilm development and its metabolism is crucial for biofilm formation in *P. aeruginosa* (Kang, Turner, & Kirienko, 2018; Kang & Kirienko, 2018).

Conclusion

In conclusion, our results show that the interaction of one *P. aeruginosa* strain with another in co-cultures promotes a decrease in sessile cell count that is partially maintained in monocultures of the first strains grown with metabolites of the second. Such metabolites also interfered with expression of genes related to iron acquisition, suggesting that the decreased biofilm in stainless steel coupons might be due to reduced iron uptake by the cells. Since no significant difference was observed in the expression of the three QS regulators analyzed, the pathway these molecules interfere with was undetermined, but this mechanism of action revealed that intraspecies interactions might influence *P. aeruginosa* biofilm formation, which might be an interesting approach for better understanding the species lifestyle.

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