Distinct stress responses to pyocyanin by planktonic and sessile Staphylococcus aureus UFPEDA 02 and Escherichia coli UFPEDA 224

Respostas distintas ao estresse causado pela piocianina em células planctônicas e sésseis de Staphylococcus aureus UFPEDA 02 e Escherichia coli UFPEDA 224

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ABSTRACT
Antimicrobial activity of pyocyanin against competing organisms of Pseudomonas aeruginosa is related to the oxidative stress that the compound promotes in susceptible cells. The objective of this work was to produce, extract and verify the activity of pyocyanin in planktonic and sessile forms from clinical strains, Staphylococcus aureus UFPEDA 02 and Escherichia coli UFPEDA 224. About 600 µg/mL of pyocyanin were

obtained. The planktonic cells were highly sensitive. The MIC determined for S. aureus UFPEDA 02 and E. coli UFPEDA 224 were 18.75 and 37.5 µg/mL, respectively. The pyocyanin demonstrated biocidal effect against S. aureus UFPEDA 02. On the other hand, pyocyanin was not active in either sessile strain. The presence of the pigment allowed a greater adherence of the strains, forming more robust biofilms compared to the control. S. aureus UFPEDA 02 and E. coli UFPEDA 224 presented moderate and high hydrophobicity, respectively. Glass and dolomite surfaces were tested in the in vitro biofilm test. Both strains formed the biofilm better on the dolomite surface, obtaining a cell concentration (MPN/cm^2) in the order of 3 log units after 48h of incubation.

Key-words: Biofilm, Negative microbial interactions, Pyocyanin.

RESUMO

A atividade antimicrobiana da piocianina contra organismos competidores da Pseudomonas aeruginosa está relacionada ao estresse oxidativo que o composto promove nas células susceptíveis. O objetivo deste trabalho foi produzir, extrair e verificar a atividade da piocianina nas formas planctônica e séssel a partir de linhagens padrão, Staphylococcus aureus UFPEDA 02 e Escherichia coli UFPEDA 224. Foram obtidos cerca de 600 µg/mL de piocianina. As células planctônicas foram altamente sensíveis. A CIM determinada para S. aureus UFPEDA 02 e E. coli UFPEDA 224 foi de 18,75 e 37,5 µg/mL, respectivamente. A piocianina demonstrou efeito biocida contra S. aureus UFPEDA 02. Por outro lado, a piocianina não foi ativa às linhagens na forma séssil. A presença do pigmento permitiu uma maior aderência das bactérias, formando biofilmes mais robustos em comparação com o controle. S. aureus UFPEDA 02 e E. coli UFPEDA 224 apresentaram uma hidrofobicidade moderada e alta, respectivamente. Cupons de vidro e dolomita foram utilizados no teste in vitro de formação do biofilme. Ambas as linhagens formaram melhor o biofilme na superfície da dolomita, obtendo uma concentração celular (MPN/cm^2) na ordem de 3 unidades logaritimicas após 48h de incubação.

Palavras-chave: Biofilme, interações microbianas negativas, piocianina.

1 INTRODUÇÃO

Bacterial population interact with each other through three ecological relationships: 1. Positive (commensalism, proto-cooperation and mutualism), 2. Negative (mutual inhibition by competition, competition for the use of resources, amensalism and parasitism), or 3. Neutral, when there is no influence between populations (Godsoe et al., 2017). When competing species coexist in the same environment, antagonistic relationships are established naturally, based on competition for nutrients, direct interactions between competitors or induction of resistance (Molina-Santiago et al., 2017; Lopes et al., 2011). Thus, microbial competition is determined by obtaining an advantage over the opponent and in the genetic transfer (Hibbing et al., 2010).

P. aeruginosa is a non-fermenting, aerobic, ubiquitous Gram-negative rod, member of fluorescent pseudomonads and measuring 0.5 x 1.5 µm (Viana et al., 2017).

In addition, it exhibits great metabolic versatility (El-Fouly et al., 2015), giving it qualities of resistance to environmental pressures, as well as superiority over other competing organisms (Scott-Thomas et al., 2010), including pathogens, Escherichia coli (Vasconcelos et al., 2010) and Staphylococcus aureus (Hotterbeekx et al., 2017).

One of the most important competition strategies for P. aeruginosa is the synthesis of pyocyanin, a bright blue phenazine pigment, synthesized exclusively by 90-95% of strains (Gonçalves and Vasconcelos, 2021). The role of pyocyanin in inhibiting competing microorganisms is associated with oxide-reduction reactions, with the accumulation of reactive oxygen compounds (Bahari et al., 2017).

Microbial susceptible microbes can use biofilm formation as one of their resistance strategies. These structures are highly complex morphofunctional associations, characterizing the preferential life style of several microorganisms (Satpathy et al., 2016). This organization makes it possible to maintain the population in oligotrophic environments and/or under competition (Huynh et al., 2012), ensuring stability through the exchange of nutrients, metabolites and oxygen, in a sophisticated cell signalling system (Monds and O'Toole, 2009). In addition, the formation of biofilm allows horizontal gene exchange, resulting in increased resistance to substances with antimicrobial properties (Costa et al., 2014). The aim of the present work was to produce and verify the action of pyocyanin in the adhesion of two pathogens with epidemiologic importance (Martins et al., 2019).

2 MATERIALS AND METHODS

2.1 MICROORGANISMS

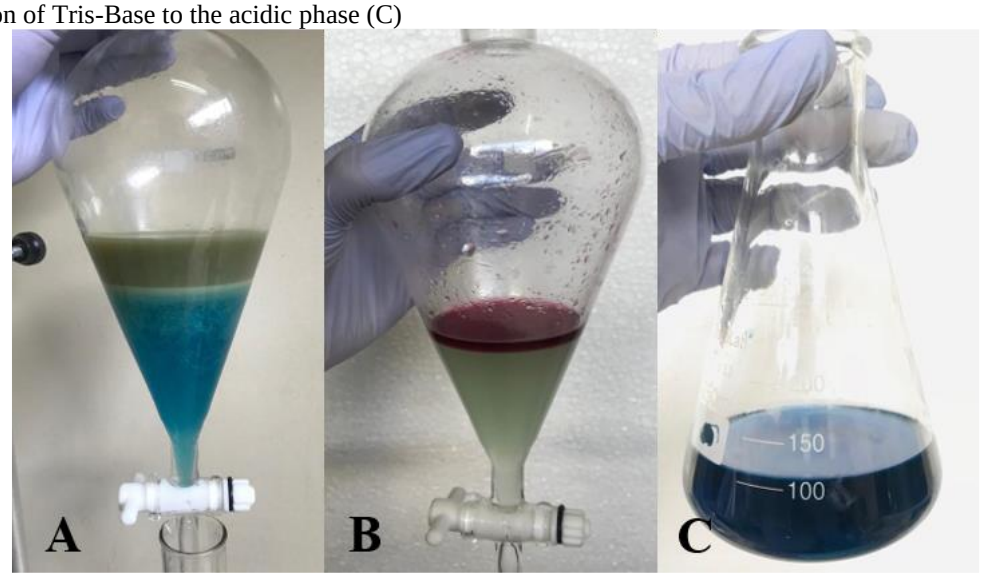
Two wild specimens of P. aeruginosa, TGC02 and TGC04 (Cavalcanti et al., 2019) were used for the production of pyocyanin (SisGen register #A6D0C2F). For in vitro biofilm tests, the standard strains Escherichia coli UFPEDA 224 and Staphylococcus aureus UFPEDA 02 were used. The inoculants were prepared in 0.85% NaCl solution from cells of recent culture, with standard turbidity by tube #1 of the MacFarland scale.

2.2 PRODUCTION AND EXTRACTION OF PYOCYANIN

The TGC02 and TGC04 isolates were subcultured in nutrient agar for 24h at 30ºC and further incubated in King A broth by using inoculating loop 10 µL. The broth was modified by the addition of 2% (m/w) of beer malt bagasse. The flasks were incubated at 29±1°C for 96h at 150 rpm (Oliveira et al., 2019). Pyocyanin was extracted using the

method described by a previous study, with modifications (Hassani et al., 2012). Briefly, 50 mL of the cell-free culture medium was mixed with 10 mL of chloroform. After vortexing for 1 minute, the chloroform+pyocyanin fraction (blue) was separated, and then 5 mL of HCl 0.2 mol/L were added and neutralized with Tris-Base buffer 0.1 mol/L. The procedure was repeated 3 more times, resulting in approximately 600 µg/mL of pyocyanin at the end (Figure 1).

Figure 1 Steps for the extraction of pyocyanin: extraction in chloroform (A), addition of HCl (B) and addition of Tris-Base to the acidic phase (C)



2.3 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

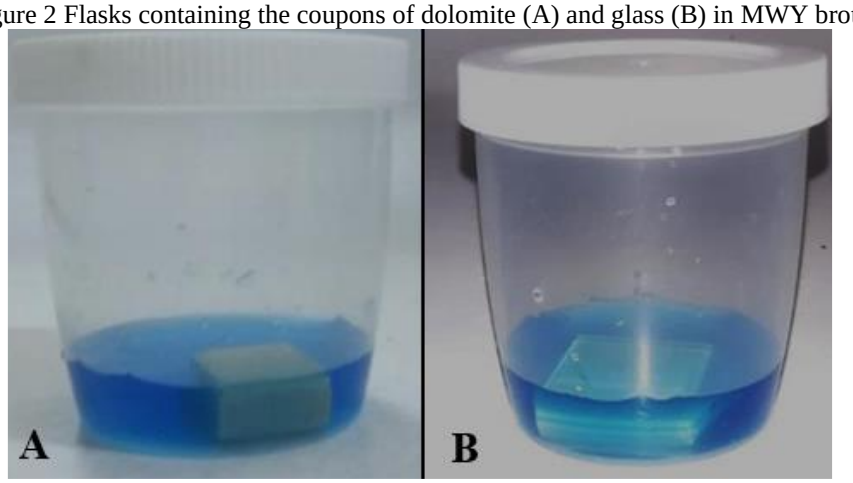
The test was conducted using the microdilution technique (Hadacek and Greger, 2000). The wells were filled with 150 µL of nutrient broth, 150 µL of pyocyanin solution (ranged 300 to 18.75 µg/mL) and 20 µL of the inoculum. The microplates were incubated for 24h at 30°C. MIC was defined as the lowest concentration of pyocyanin that inhibited microbial growth, verified by visual inspection of turbidity, and compared to control Pffaler et al., (1995). Pyocyanin activity was interpreted as high (50>MIC<500), moderate (500>MIC<1500) or weak (MIC>1500 µg/mL) (Sartoratto et al., 2004). Assessment of bactericidal or bacteriostatic effect was performed by determining the Most-Probable-Number (APHA, AWWA and WEF, 2012).

2.4 IN VITRO ASSAY OF BIOFILM FORMATION

The test was performed using the method described by Almshawit et al. (2014) to assess the disturbance effect of pyocyanin on the adhesion and formation of the E. coli

UFPEDA 224 and S. aureus UFPEDA 02 biofilm on the surface of 1 cm^2 coupons of dolomite (Legato, Rio de Janeiro, Brazil) and glass (Sincenet, Joinville, Santa Catarina, Brazil). The coupons were aseptically immersed in flasks containing 10 mL of a sterile mineral water solution containing 0.5 g/L of yeast extract (MWY) (Viana et al., 2017) and 37.5 µg/mL of pyocyanin (Figure 2). Then 1 ml of the inoculum was added. The flasks were statically incubated at 30ºC for 48h (BOD, SL–200). For the control, pyocyanin was not added. After, the coupons were aseptically removed and the organic matter deposited on the surfaces in contact with the aqueous phase, then scraped into a container containing 10 mL of NaCl 0.85%. After homogenization, 1 ml was transferred into a test tube containing 9 ml of NaCl 0.85% and the Most-Probable-Number per cm^2 was determined.

Figure 2 Flasks containing the coupons of dolomite (A) and glass (B) in MWY broth



2.5 CRYSTAL VIOLET TEST

The test was carried out in microtubes containing 1000 µL of nutrient broth. Then, 5 µL of an inhibitory concentration of pyocyanin was added together with 10 µL of the inoculum and incubated for 48 h at 30ºC. Afterwards, the supernatant was discarded and the walls of the microtubes were washed 3-5 times with tap water and then dried for 1 hour. Subsequently, 1500 µL of 1% crystal violet dye was added and after 20 min, the dye was discarded and its excess on the walls, removed with tap water. After drying, the microtubes were filled with 1500 µL of absolute ethanol and after 30 minutes rest, the absorbance of the crystal-ethanol solution was determined at 590 nm (Balasubramanian et al., 2012). The percentage of adherence was calculated using the formula [(ODC - ODT) ÷ ODC x 100], where ODC is the average of the optical density of the control in nutrient broth and ODT, the average of the optical density of the treatment (Pagano et al.,

2004). Cell adhesion was classified as poor when <40%; moderate, when ≤ 40 and ≥80%; and strong, when> 80% (Rodrigues et al., 2010). Pyocyanin was not added to the control.

2.6 MICROBIAL ADHESION TO HYDROCARBON (MATH) ASSAY

An adaptation of the MATH technique was used for assessing hydrophobicity (Tyfa et al., 2015). Suspensions of *E. coli* UFPEDA 224 and *S. aureus* UFPEDA 02 were prepared in PBS buffer (pH 7.2), so that the absorbance varied from 0.4-0.6 at 600 nm (Ai). Then, in a microtube, 0.5 ml of xylene was mixed with 2.5 ml of the suspension and after vigorous vortexing for 1 minute and resting for 10 minutes at 25ºC, the microtubes were again stirred for 1 minute and left to rest for another 10 minutes. After 10 and 60 minutes, the absorbance of the solution was measured at 600 nm (Af). The percentage of hydrophobicity was determined using the formula: [(1–Ai)÷Af] x 100. The hydrophobicity was classified as strong (> 50%), moderate (20-50%) or weak (<20%).

2.7 STATISTICAL ANALYSIS

All tests were performed in triplicate. The results of the hydrophobicity and in vitro biofilm tests were expressed by the mean value and its standard deviation. The results of the crystal violet test were expressed by the mean value and its standard deviation, followed by the unpaired T test.

3 RESULTS AND DISCUSSION

Initially, the strains were tested for susceptibility to pyocyanin. The pigment activity was high, due to the MIC, being respectively, 18.75 and 37.5 µg/mL, against S. aureus UFPEDA 02 and E. coli UFPEDA 224. The pyocyanin MIC produced a bacteriostatic effect in E. coli UFPEDA 224 and bactericidal for S. aureus UFPEDA 02.

Thus, the tests were continued with pyocyanin in a concentration of 37.5 µg/mL. All in all, at this concentration, there was the formation of E. coli UFPEDA 224 and S. aureus UFPEDA 02 biofilms on the tested surfaces. There was a significant difference from the control under all conditions tested, with the exception of S. aureus UFPEDA 02 in the presence of the pigment (Table 1).

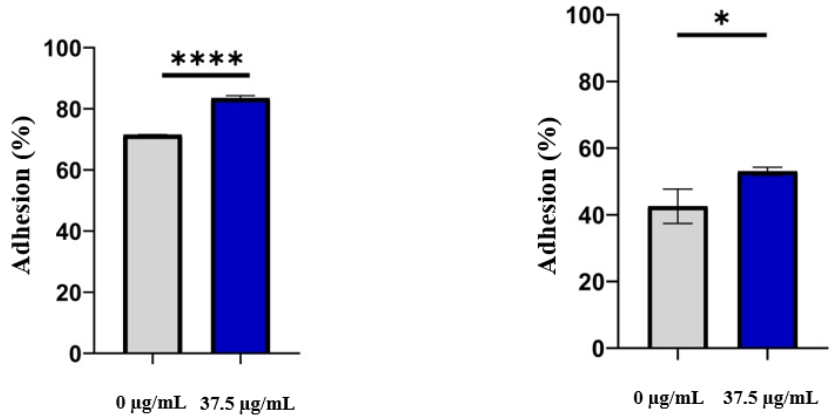
Table 1 – Cellular density on the surface of glass and dolamite coupons (10^3 NMP/cm^2) in the presence of 37.5 µg/mL of pyocyanin*

Strains	Surfaces			
	Dolomite		Glass	
	Treatment	Control	Treatment	Control
S. aureus UFPEDA 02	3.4	60.5	17.9	23.0
E. coli UFPEDA 224	50.1	92.9	20.3	58.5

*hydrophobicity: E. coli UFPEDA 224 (61.9%, strong) and S. aureus UFPEDA 02 (27.3%, moderate)

Figure 3 illustrates the percentage of adhesion of the two strains, in the presence of 37.5 µg/mL of pyocyanin. Compared to the control, the presence of the pigment allowed a greater adherence in both strains. For S. aureus UFPEDA 02, strong adherence was observed, representing a significant improvement of around 43%, compared to the control. The absorbance measure was 0.379 (control 0.222).

Figure 3 – Percentage of adhesion of Staphylococcus aureus UFPEDA 02 (left) and Escherichia coli UFPEDA 224 (right) in the presence of pyocyanin (**** p<0,0001 e *p<0,05)



Interestingly, pyocyanin did not disturb S. aureus UFPEDA 02 pioneer cells, while E. coli UFPEDA 224 appeared to be more sensitive. However, its biofilm was formed. By assuming that when the absorbance value in the treatments is above the cutoff, that is, three times the absorbance measurement of the culture broth used in the test, the viability of the pioneer cells is confirmed, suggesting the ability of the strain to adhere. The closer to or below the cutoff value, the weaker the adhesion, which is indicative of the antibiofilm potential of the tested substance. In this study the cutoff was 0.189.

It should be noted that the absorbance value of E. coli UFPEDA 224 in the control was less than the cutoff value, 0.111 (treatment 0.134); this may have reflected in the apparent sensitivity of the strain in the test. Despite this, a moderate adhesion of E. coli

UFPEDA 224 was observed, with a significantly stronger adhesion, approximately 20%, compared to that obtained with the control.

The in vitro production of pyocyanin occurs between 48-72h after incubation, under ideal conditions of temperature (29±1ºC) and agitation (150 rpm) (Agrawal and Chauhan, 2016). The pigment production coincides with the beginning of the stationary phase, a growth stage dependent on the generation time of the isolates, which under controlled conditions, ranges 3-6h (Tamagnini and Gonzales, 1997). Additionally, the in vitro production of pyocyanin is based on the reduction of the energy status of P. aeruginosa, achieved by reducing the concentration of nutrients, resulting in a decrease in the population growth rate and an increase in the pigment concentration (Whooley and McLoughlin, 1982).

The supplementation of the medium with beer malt bagasse to the King A broth significantly increased the production of pyocyanin, allowing a yield of approximately 600 μg/mL. This value was dramatically higher when compared to previous studies, whose concentration of pyocyanin ranged 9-92 μg/mL in media other than King A broth (Elbargisy, 2021; Gahlout et al., 2021; Devnath et al., 2017; Barakat et al., 2014; Gharieb et al. 2013). In addition, when using King A broth, with the regular or modified formulation in terms of its constituents without additional substances, the maximum concentration of pyocyanin yielded 80 μg/mL (Hassani et al., 2012; El-Shouny et al., 2011). However, it is noteworthy that the amount of pyocyanin produced in vitro may be variable for the same strain submitted on different occasions under identical conditions of incubation. This is related to the stresses that cells are exposed to in the laboratory (Viana et al., 20185).

The premise that the biocidal/biostatic activity of pyocyanin is concentration-dependent was conceived almost 4 decades ago (Baron et al., 1989). In addition, the effect is related to the species involved (Pal et al., 1998). In the planktonic phase, S. aureus UFPEDA 02 and E. coli UFPEDA 224 were more sensitive to pyocyanin and presented a biocidal activity against S. aureus UFPEDA 02. The activity of pyocyanin on S. aureus occurs by blocking the respiratory chain (Voggu et al., 2006). However, there are reports that other intermediary phenazines produced by P. aeruginosa may also be involved in this mechanism (Machan et al., 1991). On the other hand, pyocyanin participates in the depletion of the oxygen supply in E. coli, due to the generation of peroxides and the diversion of electron flow, resulting in a significant cytotoxic effect (Andrade et al., 2016).

S. aureus UFPEDA 02 and E. coli UFPEDA 224 formed biofilm, suggesting that for the sessile status, even though pyocyanin remains active, the biofilm protected the cells. The presence of pyocyanin exerts a selective pressure on sensitive bacteria, which need to respond quickly to stress, with the formation of a robust biofilm in the shortest time, a crucial mechanism to ensure persistence (Costa et al., 2014). However, adherence is the most critical stage for the establishment of biofilm under stressful conditions (Lin et al., 2010; Banning et al., 2003).

Stress response has been studied to some extent in S. aureus. The adhesion process of S. aureus is fast, occurring in less than three hours, especially under favourable conditions of temperature, pH, space and nutritional availability (Millezi et al., 2012). In addition, some S. aureus phenotypes are selected, especially cells that exhibit defects in the electron transport chain. Thus, S. aureus may coexist with P. aeruginosa, in the presence of the pigment (Noto et al., 2017; Hoffman et al., 2006). This may justify the 43% improvement in the adhesion. The increase in S. aureus biofilm compared to the control has already been observed in previous studies (Balasubramanian et al., 2012; Khare and Arora, 2011). However, not in the same magnitude achieved in this work.

The adhesion of E. coli UFPEDA 224 was approximately 20% better, compared to the control. One of the strategies of E. coli stress response is to produce indole to reduce the antagonistic activity of P. aeruginosa (Arruda et al., 2020). In addition, E. coli produces more biofilm as a defensive response against several stresses, including those of an oxidative nature. Stress conditions also significantly induce the expression of the ycfR gene, which encodes a putative outer membrane protein YcfR, causing changes in hydrophobicity and acting effectively against the formation of the biofilm (Zhang et al., 2007).

Microbes with greater hydrophobicity adhere better to surfaces with hydrophobic properties and the same occurs for those with hydrophilic properties. Hydrophobic surfaces, however, appear to be more susceptible to colonization, explaining the greater adherence to dolomite than to glass. In addition, there is a microbial preference for more porous surfaces (Bos et al., 2000; Chang and Merritt, 1994; Scheuerman et al., 1988). This preference is due to the better adaptation of the bacteria to the microenvironment with dimensions similar to their own size, as well as by the increased contact area present on these surfaces (Schierholz et al., 2000).

Ionic strength and pH also interfere with the characteristics of the bacterial surface and the electrical charge varies according to the species (McWhirter et al., 2002; Bunt et

al., 1995). In aqueous media, most planktonic cells tend to have a negatively charged surface (Poortinga et al., 2001). The more hydrophobic the cell, the greater the adhesion to a certain surface (Collin et al., 2020). This may be achieved by the expression of some structures, such as flagella, pili, as well as by the presence of polar functional groups on the membrane, for example, hydroxyls, phosphates, carboxyls and teicoic acid (Boari et al., 2009).

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