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Synergistic Effect of Tryptophan and Erythromycin on *Pseudomonas Aeruginosa* Biofilm Structure and Dispersal

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Pseudomonas aeruginosa is an opportunistic pathogen that is responsible for diseases such as cystic fibrosis (CF) in immunocompromised individuals. Outcome of CF is abnormally thickened mucus that causes problems in patient's respiratory system. This condition affects 2.5 million people in the UK alone with a high rate of mortality by the age of 40. Various methods of treatment to manage CF by dispersion and disassembly of biofilm have been intensively researched. Since as much as 80% of human bacterial infections are biofilm-associated, many researchers have begun investigating therapies that specifically target the biofilm architecture, thereby dispersing the microbial cells into their more vulnerable, planktonic state. Amongst the current methods of controlling CF, biofilm dissociation by the use of a combination of an amino acid and an antibiotic has been investigated in this research. During this study, biofilm formation by *P. aeruginosa* PAO1 was observed under aerobic conditions in Luria Broth and M63 minimal media at 37°C for a period of 24 hours. The cultures were treated with two isomeric forms of tryptophan at different concentrations (1 mM, 4 mM, and 8 mM). Dispersal and dissociation of the cells in all cultures were investigated and compared with the control after 24 hours. The D isomer of tryptophan at concentrations of 4 mM and 8 mM showed higher rate of dispersion in comparison to the L isomeric form and the control. The effect of tryptophan varied with the medium that was used for biofilm growth. Extracellular polymeric substances (EPS) were extracted from the treated and untreated biofilm and quantified for their main components. Biofilm treated with tryptophan and erythromycin resulted in nearly 70% loss of EPS components in comparison with the control after 5 days of growth.

1. Introduction

1.1 *Pseudomonas aeruginosa* and biofilm formation

Pseudomonas aeruginosa possess the ability to communicate within the bacterial population through the secretion of chemical signals called autoinducers. Bacteria are able to use a wide range of molecules for signalling purposes such as fatty acids, peptides, and *N*-acylated homoserine lactones (AHLs). These are called autoinducer 2 (AI-2). As the concentration of an autoinducer increases the cell density increases relatively until it reaches a threshold concentration. The phenomenon by which bacterial communication takes place is known as quorum sensing (QS) (Hirakawa and Tomita, 2013; Palmer *et al.* 2013).
QS controls processes such as bioluminescence, sporulation, antibiotic production, virulence factors, and biofilm formation (Rutherford and Bassler, 2012). Through expression of the specific signals, *P. aeruginosa* can control its virulence factors (Rice *et al.* 2008). Factors such as elastase, proteases, rhamnolipids and exotoxin A, pyoverdin, pyocyanin have been vastly studied (Rutherford and Bassler, 2012).
Biofilm formation develops in series of stages. Firstly, the attachment of planktonic cells on a surface takes place where van der Waals forces form a weak reversible adhesion. Secondly, cells anchor themselves to the surfaces using pilli, at this stage cells begin to form a monolayer. This creates small clusters and upon reaching certain cellular density the biochemical signal molecules are produced and released, for example *N*-acylhomoserine lactone (AHLs). With the progression of colonisation, these form irreversible structures

resistant to antimicrobials and disinfectants (Sharma, *et al*. 2014). After 8-24 hours, colonies form a stable attachment to the surface and to each other through secretion of extracellular polymeric substances (EPS) material that forms a sticky layer containing eDNA, proteins, carbohydrates and other cellular debris, which in turn protects colonised cells and promotes further growth of colonies (Pan *et al*. 2010). Changes in gene expression takes place upon the signalling molecules reaching the required threshold level. This in turn brings a phenotypic change from planktonic to biofilm forming sessile cells. The positive feedback loop is formed through synthesis of proteins (autoinducers) that is directly related to expression of QS. Finally, colonies reach maturation that leads to further dispersion of biofilm (Zalewska-Piatek *et al*. 2013).

1.2 Medical relevance of biofilm formation

Pseudomonas aeruginosa is an opportunistic bacterium that can cause acute and chronic infections especially in immunocompromised individuals. The most common infection that *P. aeruginosa* is responsible for is cystic fibrosis (CF) (Sharma, *et al.* 2014). CF is characterised by lowered pulmonary function and increased mortality (Rutherford and Bassler, 2012). Through the expression of QS by *P. aeruginosa,* thick condensed layer of biofilm forms, blocking pulmonary organs and as a result it leads to respiratory failure (Hirakawa and Tomita, 2013). In the era where antibiotic resistance is a widespread problem, biofilm produced by number of microorganisms have been recognised as a potential target in development of new antimicrobial agents (Hirakawa and Tomita, 2013).

P. aeruginosa express high antibiotic resistance to multiple antibiotics classes and it is difficult to eradicate (Poole, 2011). Resistance acquired by *P. aeruginosa* is due to chromosomal mutation and acquirement of resistance genes through horizontal gene transfer (Poole, 2011). Scientific literature describes number of quorum sensing inhibitors already in use. Despite the fact that *P. aeruginosa* is difficult to eliminate, studies show that novel therapies are being developed that combine amino acids and antibacterial compounds (She *et al*. 2015) to combat the rise in antibiotic resistance. Furthermore, scientific work suggests that macrolides class of antibiotic negates the effect of QS induced biofilm formation by *P. aeruginosa* (Tsang *et al.* 2003). Hence, for the purpose of this study, erythromycin was chosen.

It has been reported that certain amino acids (Sanchez *et al*. 2013), cause biofilm dispersal and disassembly in bacteria, while lacking significant toxicity. The idea of targeting human pathogen such as *P. aeruginosa* with naturally occurring amino acid - tryptophan is a highly attractive idea. For the purpose of this study, dextrorotatory (D-) and laevorotatory (L-) isoforms of tryptophan have been chosen and three different concentrations (1 mM, 4 mM, 8 mM) selected to determine whether these concentrations have any effect on dispersion of *P. aeruginosa* PAO1 biofilm. Following that, combination of two isoforms of one concentration (the most effective one) of tryptophan and antibiotic erythromycin against *P. aeruginosa* PAO1 biofilm will be performed. The dispersal and inhibition activity of the biofilm dissociation treatment will be investigated.

2. Materials and Methods

2.1 Strain and media

Pseudomonas aeruginosa PAO1 strain was obtained from bacterial culture collection at University of Westminster, London, UK. Mueller Hinton Agar (MH) was used for the maintenance of the *P. aeruginosa* PAO1 working stock. M63 minimal medium (pH was adjusted to pH 7 after addition of 1mL-L 1M $MgSO_4$ and 10 mL-L of 20% glycerol) Luria-Bertani Broth (LB) (pH was adjusted to 7.4) were used for biofilm growth.

2.2 Biofilm quantification by Crystal violet assay

For the determination of biofilm formation and subsequent application of biofilm dissociation treatment, 96-microtiter plates were used. The general method of growing, staining and quantifying of biofilm was followed as described by O'Toole (2011). Each experiment was incubated in the same static conditions; 24 hours at 37°C.

2.3 Extraction of EPS from biofilm

The extraction method followed was as published by Staats *et al*. (1999) with a slight modification. After the specific incubation time, the biofilm was scraped from the glass well walls using sterile techniques. The aliquots were then transferred to Eppendorf tubes and centrifuged at 3500 rpm for 30 min. After centrifugation, the supernatant containing dissolved carbohydrates and suspended protein was analysed after separation of bacterial cells.

2.4 Colorimetric quantification of Carbohydrates and Protein content of EPS

Bradford Assay was used to quantify proteins and the phenol-sulphuric acid assay as described by DuBois *et al.* (1956) was used to quantify carbohydrate content of the EPS extracted from the biofilm.

2.5 Tryptophan and Erythromycin

D- and L- isoforms of tryptophan were purchased from Sigma-Aldrich, UK. A working stock of 100 mM was prepared and diluted in Luria broth and M63 medium as required. Erythromycin was purchased from Sigma-Aldrich, UK. A stock solution of 40 µg/mL was prepared and used as required.

2.6 Biofilm dispersal treatment

First set of experiment was quantifying the effect of three different concentrations (1 mM, 4 mM, 8 mM) of D- and L- isoforms of tryptophan on biofilm formed by *P. aeruginosa*. Plates were incubated at 37°C for 24 hours. Biofilm staining and quantification was performed as described previously. Second set of experiments examined the effect of erythromycin (4 µg/ml) combined with the mixture of D- and L- form of tryptophan on biofilm formation. Plates were left in the incubator at 37°C, 24 hours. Biofilm staining and quantifying was followed as described previously.

2.7 Statistical analysis

All experiments were carried out in triplicates and the statistical significance of each result was determined by one-way analysis of variance (ANOVA) through calculation of standard deviation from the mean and use of standard error.

3. Results and discussion

3.1 Tryptophan dispersal assay

All of the constituents of the assay were loaded at the inoculation phase. The plates were then incubated for a period of 24 hours. After which, the biofilm was quantified by the crystal violet assay. Biofilm formation was tested with three increasing concentration (1 mM, 4 mM, and 8 mM) of the two isoforms of tryptophan.

Growth in LB and M63 media are presented in Figure 1. The highest inhibition of biofilm formation was noted in D- form in 4 mM and 8 mM in comparisons to the L- form and the control. As the highest inhibition of biofilm was seen in samples containing D- isoform of tryptophan, this isoform was investigated further.

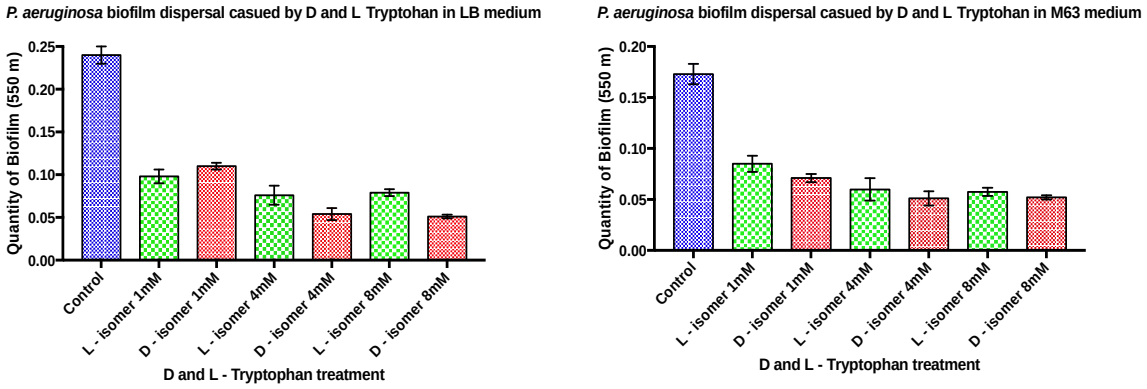

Figure 1. Effect of D- and L- Tryptophan at various concentrations on biofilm formation and dispersal in comparison to the control after a period of 24 hours in LB medium (p <0.05; p=0.001) and M63 medium (p<0.05; p=0.001)

The EPS extraction performed showed a decrease in carbohydrates content after treatment with 4 mM and 8 mM tryptophan respectively. Additionally, the protein content was found to be lower than the control in 4 mM and 8 mM treatments. All the factors were found to inhibit formation of biofilm; this was most likely due to the media used. However, despite the minimal media factor, the high decrease was noted in 8 mM concentration in both D- and L- isoforms with similar decrease outcomes in 4 mM tryptophan.

3.2 Quantification of biofilm components

Extraction assay performed on D- isoform samples for carbohydrates and proteins resulted in confirmation of the findings stated above. The 8 mM treatment showed ~80% decrease in carbohydrate content of the biofilm, while the 4mM treatment showed ~60% decrease. The proteins level decreased similarly with a ~ 70% decrease after treatment with 8 mM and ~80% after treatment with 4 mM tryptophan. These findings correlated with the absorbance readings and suggests that biofilm forms weaker structures on minimal media.

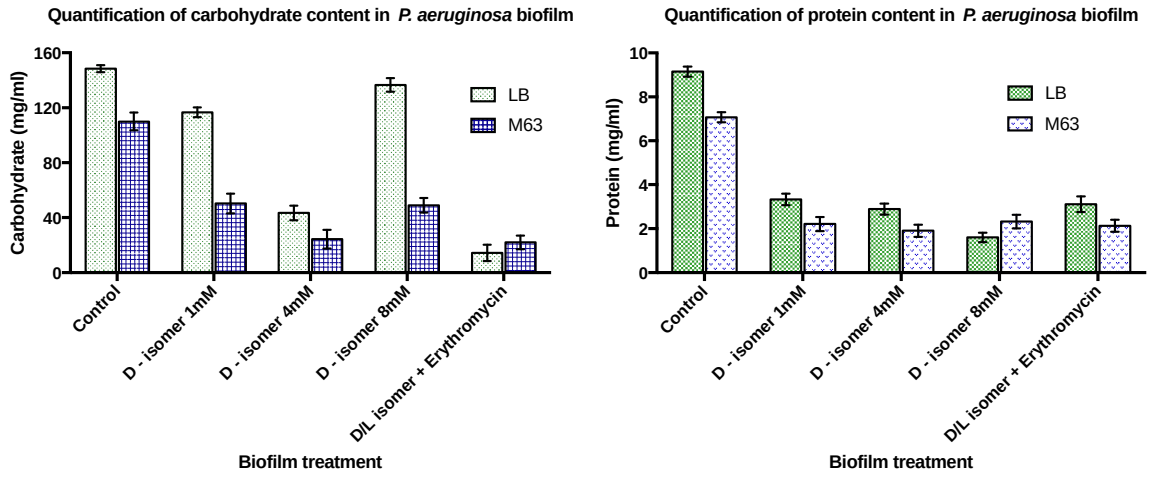

Figure 2. Quantification of carbohydrates and proteins extracted from P.aeruginosa biofilm after treatment with isomers of tryptophan and erythromycin and combination treatment.

4. Discussion

Biofilm development responds to the stress experienced by cells towards environmental changes, such as growth medium, where nutrients are limited, temperature, pH, and upon administration of antibiotics (Landini, 2009). Therefore, two different media were tested to express stress factors on *P. aeruginosa* PAO1. Carbon source, salts, and metals ions influence the structure, size and growth rate of biofilm (Zalewska-Piatek *et al*. 2013). M63 minimal medium consist of glycerol, which can serve as a carbon source for the growth of *P. aeruginosa*. Nitrogen, phosphorus and trace metals represents salts requirement for bacteria. Addition of magnesium sulphate promotes the enzymatic reaction required for DNA replication. Luria-Bertani medium containing tryptone that provides amino acids, yeast extract delivers vitamins and trace elements, while sodium is responsible for osmotic balance.

The use of naturally occurring amino acid as a treatment of infection caused by bacteria is an interesting idea. Amino acids do not express any toxicity to the host while bacteria do not acquire resistance towards amino acids. Numerous Gram-positive and Gram-negative bacteria produce D- amino acids such as D- alanine and D- glutamic acid in a stationary phase and according to studies, D- amino acid are embedded in the peptidoglycan layer found in cell wall of bacteria (Barreteau, 2008). However, the amino acids are not produced in high volume to supress biofilm formation or cause biofilm dispersion (Kolodkin-Gal *et al.* 2010; She *et al*. 2015). Brandenburg *et al.* (2013) argues that some bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms were sensitive to amino acid and caused partial inhibition of biofilm formation. During this experiment, D- and L- isoforms of tryptophan reacted differently on formed biofilm but not on cells morphology.

Tryptophan caused dispersion and dissociation of biofilm. Furthermore, biofilm formed differently in different media that were used. *P. aeruginosa* PAO1 grown on LB, a rich media, formed a thick biofilm. However, when exposed to the L- isoform tryptophan, the loss in structure was noticed, while D- isoform caused higher rate of dispersion. The M63 biofilm control expressed different composition of biofilm in comparison to the LB control. According to the optical density, M63 displayed lower biofilm formation in comparison to the LB. As M63 is a minimal media and it is not as nutritious as LB, this explains the lower biofilm biomass.

The antibiotic chosen for the purpose of this study was erythromycin, due to studies reporting suppression of Gram-negative bacteria virulence factors (Kawamura-Sato, *et al.* 2000). Erythromycin belongs to macrolides family of antibiotics where its mechanism of action blocks translation by binding to the 50S ribosomal subunit, mostly in Gram- positive organisms. However, *P. aeruginosa* PAO1 being a Gram- negative is resistant to

macrolides. A high MIC (erythromycin) of 512 µg/ml was found to effect *P. aeruginosa* PAO1 in Mueller – Hinton (Morita, *et al.* 2014). Nalca *et al*. (2006) reports that low dose of another type of macrolides – azithromycin 2 µg/ml had an effect on quorum-sensing autoinducers resulting in inhibition of *P. aeruginosa* PAO1 virulence factors. Study conducted by Tsang *et al.* (2003) suggests that erythromycin in low concentrations, such as the one chosen for this study 4 µg/ml affects *P. aeruginosa* cellular morphology through mechanism other than common antibiotic method which is disturbance of ribosomal action and subsequent inhibition of protein synthesis. This study confirms the changes in morphology as claimed by Tsang *et al.* (2003) of the cells observed after treatment of erythromycin.
Another study investigating low dose of erythromycin applied to patients suffering from respiratory diseases caused by *P. aeruginosa,* resulted in overall reduction in sputum volume and increased lung function. However, the mechanism of action was confirmed not to be bacteriostatic, bactericidal or anti-inflammatory due to lack of changes in sputum densities consisting of leukocytes and pro-inflammatory mediators. These observations suggest that *P. aeruginosa* virulence factors are the main causatives of its virulence in CF (Tsang *et al.* 2003).
Biofilm produced via QS is composed of an extra cellular matrix of which, the primamry components are carbohydrates, protein and eDNA (Yu *et al*., 2015; Sharma *et al*., 2014). It is accepted that the matrix protects bacteria from antagonistic factors such as antibiotics and as a results promotes chronic infection and resistance (Mah and O'Toole., 2001). One of the possible solutions to overcome this problem is to disperse the biofilm prior to treatment as represented in this study. Dispersal agent in synergy with an antibiotic could be a viable therapeutic treatment to combat/ eradicate biofilm mediated infections without the issue of antibiotic resistance.

5. Conclusion

P. aeruginosa PAO1 produces a number of virulence factors; pyocyanin, pyoverdine, and proteases are just few of them. It is believed that these work in synergy with each other when attacking human host organ such as lung tissue (Hosseinidoust, 2013). Pyoverdine is a siderophore expressed by *P. aeruginosa* as a virulence factor. Pyoverdine growth is affected by carbon sources available to the bacteria (Chiado *et al.* 2013). Another *P. aeruginosa* virulence factor, pyocyanin is often reported to be found in high concentrations in individuals suffering from CF. Quantifying the production of the virulence factors after the treatment in this research would be beneficial to fully confirm the hypothesis. However, amino acid used most likely does not affect *P. aeruginosa* virulence factors, whereas antibiotic do. By measuring the virulence factors of *P. aeruginosa* after the treatment, this would present either increase or decrease of the virulence factors through inhibition.
Numerous studies show that *P. aeruginosa* cell morphology was modified upon treatment with different classes of antibiotics. To investigate what is causing the change or which part of bacterial cell wall component is affected, electron microscopy analysis of cell will need to be performed. Another experiment that can further validate findings from this study is minimal biofilm eradication concentration (MBEC). Sessile bacteria behave differently inside the host than in the laboratory conditions. The goal of the first part of the MBEC would be to find the concentration and time of tryptophan that disperse at least 75% of biofilm formed. Subsequently, planktonic cells can be treated with a known antibiotic that has a bactericidal effect on *P. aeruginosa*.
Poor understanding of pathogenicity of *P. aeruginosa* and increasing antibiotic resistance is directly linked with lack of effective treatment for respiratory diseases such as cystic fibrosis caused by this microorganism. The findings from this study of the effects of tryptophan alone and in combination with erythromycin on biofilm formation could potentially have important implication for future research in cystic fibrosis.

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