Motility effects biofilm formation in *Pseudomonas aeruginosa* and *Enterobacter cloacae*

Iram Liaqat[1], Mishal Liaqat[2], Hafiz Muhammad Tahir[1], Ikram-ul-Haq[3], Nazish Mazhar Ali[4], Muhammad Arshad[5] and Najma Arshad[6]

[1]Department of Zoology, Govt. College University, Lahore, Pakistan
[2]Department of Zoology, Govt. Model Town College for Women, Lahore, Pakistan
[3]Institute of Industrial Biotechnology (IIBT), Govt. College University, Lahore, Pakistan
[4]College of Nursing, Allama Iqbal Medical College, Lahore, Pakistan
[5]Department of Zoology, University of Education, Lahore, Pakistan
[6]Department of Zoology, University of the Punjab, Lahore, Pakistan

Abstract: Chronic infections caused by gram negative bacteria are the mains reasons to have morbidity and death in patients, despite using high doses of antibiotics applied to cure diseases producing by them. This study was designed to identify the role of flagella in biofilm formation Ten pure strains were collected from our lab. Morphological variation and motility assays led us to study two strains in detail. They were characterized biochemically, physiologically and genetically. Biofilm formation analysis was performed using test tube assay, congo red assay and liquid-interface coverslip assay. In order to disrupt flagella of studied strains, blending was induced for 5, 10 and 15 minutes followed by centrifugation and observing motility using motility test. Biofilm quantification of wild type (parental) and blended strains was done using test tube and liquid interface coverslip assays. 16S rRNA sequencing identified strains as *Pseudomonas aeroginosa* and *Enterobacter cloacae.* Significant biofilm formation ($p>0.05$) by was observed after 72 and 18 hours using test tube and liquid-interface coverslip assays respectively. Flagellar disruption showed that 15 minutes blending caused significant reduction in both strains, hence demonstrated that flagellar mediated motility could be a potent strategy to stabilize aggregate and invest resources for biofilm formation in *P. aeruginosa* and *E. cloacae*.

Keywords: Biofilm formation, flagellar disruption, motility, *P. aeruginosa*, *E. cloacae*

INTRODUCTION

Microbes are cosmopolitan in their distribution. They grow and flourish under suitable environmental conditions. They have different modes of living. Some of them live singly while others form colonies or associations. In most of the environments, bacteria are found in biofilms. More than 99% of all bacteria on the earth form biofilms (Nadell *et al*., 2009).

A biofilm is an aggregation of microorganisms in which the cells are attached to each other on the living or inert surfaces. These cells are embedded in a matrix which is produced by their own. This matrix is of extra cellular polymeric substances (EPS) which is also known as slime. The physical and chemical properties of EPS may be varied but basically it consists of extra cellular DNA, polysaccharides and proteins (Billings *et al*., 2015). Cells embedded in biofilm are resistant to killing by certain host defenses and various antimicrobial agents (disinfectants and antibiotics) making them almost impossible to eradicate (Bjansholt, 2013; Kouidhi *et al*., 2015).

Environmental signals including pH, temperature, nutrients and their concentrations and oxygen concentration all are also very important to induce biofilm formation. There are five stages which are involved in the development of biofilm. The first stage is the reversible adherence or attachment of bacteria to the substrate. Second stage involves the irreversible adherence due to the adhesive substances production by the bacteria. These adhesive substances are the chemical signals which are used by bacteria for communication. In this stage, the cell aggregates are formed. The third stage is called maturation I in which the cell aggregates attain a thickness of greater than 10µm due to which their motility decreases. In maturation II which is the fourth stage of biofilm formation, the biofilm reaches the ultimate thickness of more than 100mm. In the last step, the dispersal of cells takes place. They are detached gradually from the substrate and dispersed in the environment (Sharma *et al*., 2008).

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen usually present in water and soil. It is leading cause of various serious life threating infections in various immunocompromised and elderly patients including sepsis, bacteremia, wound and skin infections (Kipnis *et al*. 2006). It has contributed 57% of total nosocomial infections (Sarabhai *et al*. 2013). *Enterobacter Cloacae* comprises 40-80% of normal flora of gastrointestinal tract of population. It is widely distributed in the environment and is important cause of

**Corresponding author:* e-mail: iramliaq@hotmail.com

nosocomial infections. Like other gram negative bacteria, it is opportunistic pathogen causing serious infection in immunosuppressed, debilitated patients and neonatal intensive care units (Daniel-Hoffmann *et al.*, 2012). The pronounced ability to form biofilm, and ineffective antibiotics therapy to control diseases caused by these two strains (Bjarnsholt, 2013), make them an ideal target of interest for research focusing their disassembly.

Though extensive characterization of biofilm formation was done but molecular details are still lacking. To have understanding of molecular data, we investigated that what factors are important for biofilm initiation or adherence to abiotic surfaces. We constructed flagellar disrupted or minus strains to describe the role of flagella and motility in biofilm formation.

MATERIALS AND METHODS

Pure culture of ten strains isolated from the wounds and urinary tract was obtained from our lab. Morphologically different strains were selected and characterized upto genus level. Motility assay led us to investigate two strains for detailed study.

Morphological, biochemical Characterization and Physiological Characterization

Morphological tests like acid fast staining, gram's staining and motility test were performed to determine morphological characters of selected strains. Biochemical characterization was done performing various tests like H_2S production test, methyl red test, catalase test, citrate utilization test, voges proskauer test, tryptophan deaminase (TDA) test, indole test, urease test and denitrification tests. Physiological characterization of bacterial isolates was done by determining growth curve, optimum temperature and optimum pH (Benson *et al*., 2002).

16srRNA gene sequencing

Genomic DNA was isolated and PCR was performed to amplify 16S rRNA gene in a Techne thermal cycler under standard conditions. Invitrogen Pure Link TM kit was used to purify amplified PCR product. The amplified DNA fragments were sent to Axil scientific Singapore for sequencing. ClustalW software was used to construct the dendrograms of identified strains.

Biofilm formation assays

Biofilm formation was analysed by using three methods including 1) Congo red assay, 2) test tube assay, 3) liquid interface coverslip assay.

Congo red assay method

This method is specified for morphological characterization of biofilm-forming bacteria on congo red medium. The medium was prepared and autoclaved. Congo red stain was prepared and autoclaved separately from the rest of the medium and was poured to the medium after the medium cooled down to 55°C. Strains were streaked on plated and incubated at 37°C for 24 hours. Non-biofilm forming bacteria showed red colonies while biofilm-formers showed black crystalline colonies (Mathur *et al*., 2006).

Test tube assay

Biofilm is an aggregation of microbial cells characterized by degree of labor. Excluding control, 50µl of fresh overnight inoculum was prepared and poured in test tubes having nutrient broth. Test was performed in triplicates. The test tubes were then placed in shaking incubator at 37°C for 72, 120, 168 hours. After 72 hours, the first set of test tubes was taken out, the culture was discarded and the tubes were dried for 10 minutes at 37°C. 5ml of 0.1% crystal violet was added followed by rinsing with 0.85% NaCl. 33% glacial acetic acid was added and O.D was measured at 523 nm. The same process was repeated with the remaining two sets taken out after 120 and 168 hours. Experiment was run in triplicate (Liaqat *et al*., 2009).

Liquid-interface coverslip assay

This assay is used to determine biofilm formation by different bacteria (Mathur *et al.*, 2006 with modifications). The biofilm produced by bacteria adhered to coverslips was observed under light microscope. Pre-inoculum was prepared by growing bacteria till log phase. Culture media was prepared and dispensed into petri plates. Coverslips were placed carefully over the poured culture using properly sterilized forceps and incubated for 18, 24 and 48 hours at 37°C. Rest of the procedure was the same as mentioned above.

Construction of variants and formation of biofilm

Flagellar disruption was induced to construct variants by the process of blending. Culture media was prepared and inoculated by strains followed by incubation at 37°C for 24 hours. The culture was poured in the blender and blended for variable periods of time i.e. 5, 10 and 15 minutes. Afterwards, centrifugation was done at 12,000 rpm for 30 minutes. The supernatant was discarded. Then the pellet was streaked on the nutrient agar plates and incubated for 24 hours. Motility test was performed to check weak or minus motility. Biofilm quantification of wild type and variants was performed using test tube and liquid interface assays as mentioned above.

STATISTICAL ANALYSIS

Statistical analysis was performed applying Student 't' test. Means and SDs were calculated using Microsoft Excel software (Microsoft Corporation). A P value of <0.05 was considered statistically significant.

RESULTS

Characterization of isolated strains

The cultural, morphological and biochemical characterization of the selected isolates were performed to identify them up to species. For morphological characterization, size, shape, arrangement of the cells, spore presence or absence, acid fast and gram staining was done. Cultural and physiological characteristics include temperature tolerance, salt tolerance, IMViC test, H_2S production, nitrate reduction test, fermentation of different carbohydrates etc. All these characteristics were then analysed by comparing with description of Bergey's Manual of Determinative Bacteriology (1974). It was observed that the isolates belonged to the genus *Pseudomonas* and *Enterobacter* and provisionally identified as *Pseudomonas aeruginosa* and *Enterobacter cloacae*.

Pseudomonas aeruginosa and *E. cloacae* were physiologically characterized on the basis of growth curve, pH and temperature. Growth curves of the strains were studied for 12 hours to determine the lag, log and stationary phase. It was observed that *E. cloacae* showed lag phase of one hour followed by log phase of 4 hours and 7 hours stationary phase. In case of *P. aeruginosa*, similar pattern of growth curve was observed with the only difference that *P. aeruginosa* showed higher growth compared to *E. cloacae* (fig. 1). Both strains were subjected to a temperature range of 25-60°C and pH (3, 5, 7, 9, 11). Optimum growth was observed at 37°C with least growth at 25°C and 60°C, showing their mesophilic nature. The pH 7 was found to be the optimum one indicating that acidic pH has detrimental effect on growth of strains (fig 2a, b).

16S rRNA gene sequencing confirmed the identification of both strains upto species level as *P. aeruginosa* (KT182078) and *E. cloacae (*KT182079).

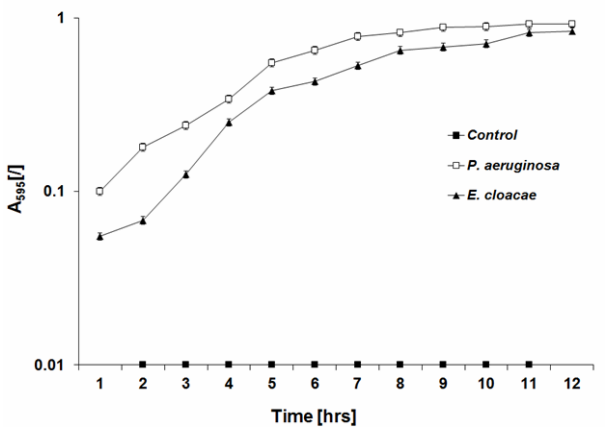

Fig. 1: Growth curve of bacterail strains. Bacteria were grown in nutrient broth for 14 hours. O.D was measured after respective time intervals at 595nm.

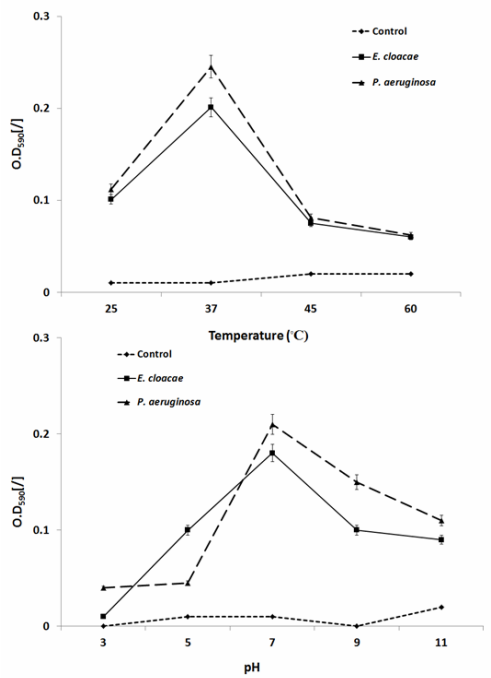

Fig. 2: Effect of temperaure and pH on growth of bacteria. Bacteria were grown in nutrient broth at (a) various temperaures (25, 37, 45 and 60°C) and (b) pH (3, 5, 7, 9 and 11). O.D was measured at 590nm.

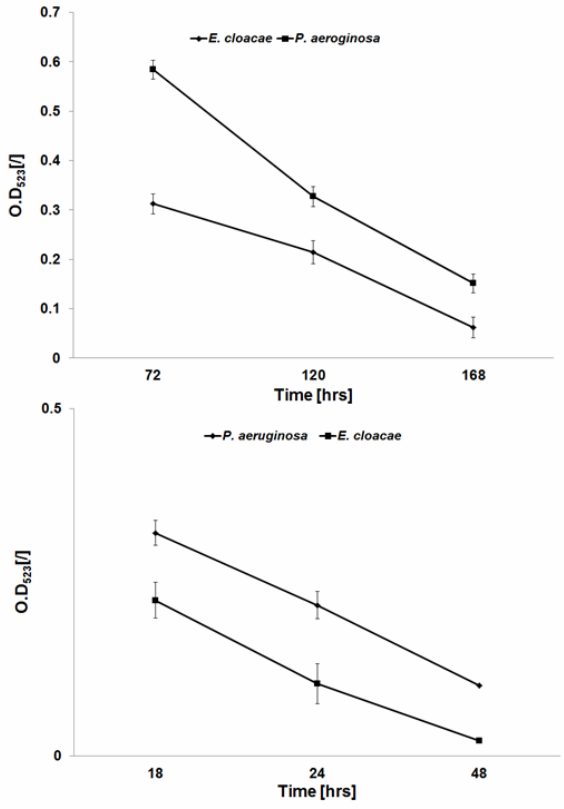

Fig. 3: Time kinetics for biofilm formation of *P. aeruginosa* and *E. cloacae* (a) Cover slip assay for biofilm formation and (b) Test tube assay for biofilm

Table 1: Congo red test for biofilm formation by *P. aeruginosa* and *E. cloacae*

Strain	Variant types	Colony color	Inference
P. aeruginosa	W8B (C)	Dark Black	Heavy biofilm formation
	W8B (1)	Black	Biofilm formation
	W8B (2)	Dull black	Less biofilm formation
	W8B(3)	Red	Absence of biofilm formation
E. clacae	SPG10 (C)	Dark Black	Heavy biofilm formation
	SPG10(1)	Black	Biofilm formation
	SPG10(2)	Dull black	Less biofilm formation
	SPG10(3)	Red	Absence of biofilm formation

formation. Both strains were grown on cover slip immersed in nutrient broth in petri plates and test tubes respectively for various time intervals. Biofilm was stained with 0.1% crystal violet. O.D was measured at 523nm.

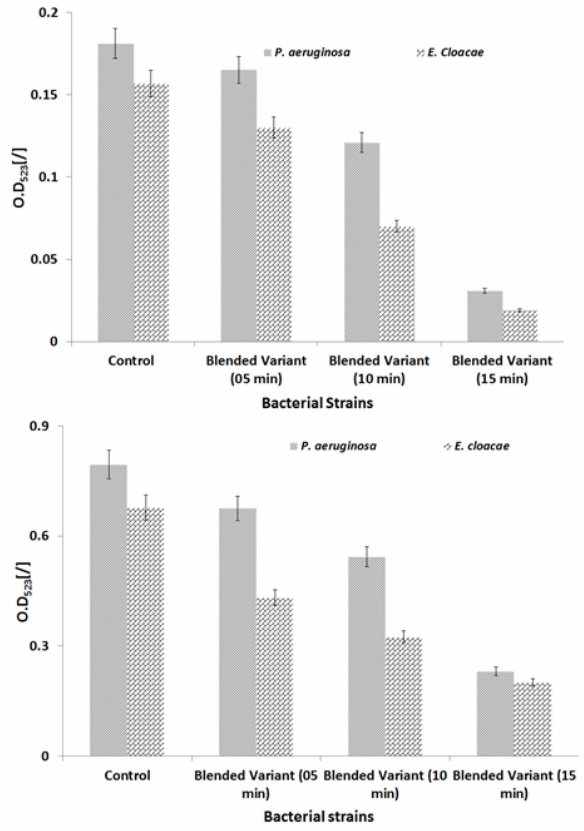

Fig. 4: Biofilm formation assays by wild type and blended variants of *P. aeruginosa* and *E. cloacae.* (a). Test tube method (b). Liquid-interface coverslip assay. Both strains and their blended variants (5, 10 and 15 minutes) were grown in nutrient broth for 72 hours. O.D was measured at 523 nm. Significant decrease ($p>0.05$) in biofilm formation was observed after 10 minutes and this decrease was highly significant ($p>0.001$) after 15 minutes. Experiment was run in triplicates.

Time kinetics of Biofilm formation

Congo red assay indicated that both strains including *P. aeroginosa* and *E. cloacae* are strong biofilm former as observed by formation of black coloured growth on congo red media (table 1).

Quantification of biofilm was done using test tube and liquid interface cover slip assay. By using test tube method, both strains produced significantly high biofilm after 72 hours however decrease in biofilm forming capacity was observed after 120 and 168 hours (fig. 3a).

Liquid-interface coverslip assay showed that *P. aeruginosa* and *E. cloacae* had high potential for biofilm formation on the glass surfaces. Results indicated that both strains produced significantly high biofilm after 18 hours and *P. aeroginosa* had high biofilm forming potential compared to *E. cloacae* (fig. 3b).

Biofilm formation by wild type strains vs blended variants

Physically disrupted variants were constructed by exposing the wild type strains to blending for 5, 10 and 15 minutes. Motility test performed afterwards confirmed the presence of weak or no motility after 10 and 15 minutes respectively. Biofilm forming potential of physically disrupted variants was studied after 120 hours in test tube assay and 18 hours in liquid interface coverslip assay, as this was the optimum time for biofilm formation in both assays in this study. Results showed that biofilm was reduced significantly by increasing blending time (fig. 4a, b). Strains exposed to blending for 5 minutes showed high biofilm formation compared to the strains exposed to 10 and 15 minutes. Significantly reduced ($p>0.01$) biofilm formation was observed after 15 minutes by flagellar disrupted variants compared with controls using both test tube and liquid interface coverslip assays (fig. 4a, b). This defect in biofilm formation observed by blended variants on various surfaces further supports our conclusion about primary role of motility in biofilm formation by *P. aeruginosa and E. cloacae.*

DISCUSSION

Attachment of Microorganisms the biotic and abiotic surface result in the formation of biofilm. Biofilm pose a universal problem in environmental and clinical settings. The fact the microbes in biofilm are highly resistant to antibiotic therapy make us to appreciate its role in

increasing clinical infections. Most of the urinary and blood borne infections are biofilm associated and their treatment require the removal or inhibition of biofilm formation (Sangita *et al*., 2012).

Total ten strains were studied following standard laboratory criteria to identify upto genus level. These include *Escherichia* spp., *Pseudomonas* spp., *Enterococcus* spp., *Streptococcus* spp., *Klebsiella* spp. and *Bacillus* spp. Organisms isolated may originate from skin of patients, tap water or other environmental sources in contact with patients. Organisms belonging to *Pseudomonas* spp., *Enterococcus* spp., *Streptococcus* spp., *Klebsiella* spp. are the common cause of nosocomial infections and survive in hospital setting despite disinfection and antibiotic therapy due to their rapid and resistant colonization on surfaces (De Rossi *et al*., 2007, Jayanthi *et al*., 2008).

The ability of bacterial strains to form biofilm is an important indicator of their pathogenicity. Among ten strains, two strains belong to genus *Pseudomonas* spp. and *Enterobacter* spp. showing best biofilm forming property were studied in detail. Time kinetics of biofilm formation was studied by three methods in this study. Congo red method showed that both wild type strains have strong ability to form the biofilm. This strong biofilm formation is the reason the *P. aeruginosa* and *E. cloacae* are cause of infection in nosocomial infections, elderly patients and are resistant to antibiotic therapy (Daniel-Hoffmann *et al.*, 2012; Bjarnsholt, 2013; Sarabhai *et al*. 2013). Other two assays were used to quantify biofilm. Our data showed that both strains had strong ability to form biofilm with varying potential OD=0.584) for *P. aeruginosa* and (O.D=0.312) for *E. cloacae* using test tube assay. Different methods are used to determine the ability of biofilm formation by the bacterial isolates. However, to assess the biofilm formation, test tube assay is considered to be the most reliable and effective method (Liaqat *et al.,* 2009). Liquid interface coverslip assay indicated that *P. aeuginosa* form maximum biofilm (O.D=0.321) compared to *E. cloacae* with lower biofilm (O.D=0.224) forming potential after 18 hours. Overall, *P. aeruginosa* produced strong biofilm compared to *E. cloacae* by both methods in this study. Rasamiravak *et al*. (2015), reported that biofilm formation by *P. aeruginosa* is the most crucial component in the causing acute and chronic infections.

Flagellum-mediated motility is considered to be important for biofilm formation by several gram-negative bacteria. Flagella have been reported to act as surface adhesions but their role in motility and thus biofilm formation has not been thoroughly investigated (Lemon *et al*., 2007). In order to induce strains with weak or minus flagella we exposed them to the blending and centrifugation at 12000 rpm for different time intervals 5, 10 and 15 minutes. Afterwards, we observed its effect on motility and on biofilm forming capability. Here we observed that blending for 5 minutes resulted in reduced motility and subsequently low biofilm formation when compared with wild type. This is in agreement with the study by Vatanyoopaisarn *et al.* (2000) who reported that flagellated cells attach more rapidly to a stainless steel surface in the absence of motility than flagellum-minus cells. Qu *et al*. (2015) recently reported that biofilm formation is mediated by flagellar motilities including swimming and swarming.

To further augment study we constructed blended variants after 10 and 15 minutes of bending followed by centrifugation. Significantly ($p>0.01$) reduced biofilm formation was observed by both strains by applying test tube assay. It has been reported previously that the flagellated wild type strains of *L. monocytogenes* attach 10-fold more to stainless steel in the first 4 h than do the flagellum-minus mutant. By 24 h, however, attachment levels are comparable (Lemon *et al.,* 2007). Likewise, similar results were obtained for biofilm formation of blended variants and wild type using liquid interface coverslip assay. This is in contrast to the findings of Lemon *et al*. (2007), who compared bacterial attachment to glass coverslips during the first 4 h of surface exposure and observed that the paralyzed-flagellum and flagellum-minus mutants showed comparably defective attachments, arguing against a role for flagella as surface adhesins. This might be due to differences in experimental protocols, hence accounting for dissimilar results. Our experiments were done in a different strains background and under different nutrient conditions directly comparing blended variants with wild type strains. Furthermore, flagellar structures play various roles in different bacteria and under different environmental conditions.

CONCLUSION

From the above discussion, it is clear that bacteria use motility to induce biofilm formation. In thinking about how flagellum-mediated motility is critical for biofilm formation in *P. aeruginosa* and *E. colacae*, we support a previously proposed model for other bacteria that the primary role of flagellum in surface-associated biofilm formation is to reduce the repulse forces that exist between bacteria and surface (Watnick and Kolter, 1999). Since motility is not required once biofilm matures. Presence of motility in mature biofilms will lead to destabilization of aggregates since extra cellular matrix production, which is necessary for biofilm formation and climax is inversely regulated by motility (Kolter and Greenberg, 2006). This emphasize the role of flagella more likely as surface adhesions, since once bacteria attach to surface they start producing extra cellular matrix. We would like to investigate that if such condition exists in *P. aeruginosa* and *E. cloacae* and how it works.

ACKNOWLEDGEMENT

This study was supported by project No.186/ORIC/18, ORIC, GC University, Lahore, Pakistan.

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