Growth in biofilm enhances potential to form new biofilm by *Pseudomonas aeruginosa*

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Abstract: Biofilm production potential of clinical isolates of *Pseudomonas aeruginosa* were investigated in this study. Isolates from blood and wound produced biofilm but wound isolates produced relatively higher amounts. Sequential passage of the *P. aeruginosa* strains in biofilm culture in trypticase soy broth (TSB) resulted in gradually increasing amount of biofilm production the by isolates whereas passage of the same isolates in planktonic culture did not result in enhanced biofilm production. Passage induced enhanced biofilm production reached maximum level at passage 3 (P-3) for the strains B-6, W-2 and W-14 and at passage 4 (P-4) for the strain B-9. These values were 64.7% 83.4% and 75.6% and 72.8 % increased biofilm production by the biofilm passaged strains in comparison to their plantonic counterparts. Ferric ammonium citrate (FAC) which inhibits biofilm production by *P. aeruginosa*, was only partially effective in reducing biofilm production by bacteria passaged in biofilm. On the other hand, FAC efficiently reduced biofilm production by cultures which were grown in planktonic state. Taken together, the results of this study indicate that growth of *P. aeruginosa* in biofilm enhances its potential to form new biofilm.
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1. Introduction

Biofilm is defined as a structured community of bacterial cells enclosed in a self-produced extracellular polymeric matrix (EPS) and adherent to an inert or living surface (Costerton *et al.,* 2001). The EPS consists mainly of carbohydrate, protein and DNA (Branda *et al.,* 2005; Mulchay *et al*., 2008). Biofilm impacts human lives in many different ways as they can form in medical, industrial and environmental settings (Daniel *et al*., 2011) Bacteria form biofilm when they transit from free floating (planktonic) state to a lifestyle in which they attach to a surface. The alternative lifestyle as a biofilm facilitates bacteria to survive in adverse environments. Bacterial cells residing in biofilm states are physiologically diverse are and less susceptible to antibacterial action of the antibiotics and clearance by immune system and hence, it is considered as a virulence attribute (Stickler, 1999; Stewart and Franklin, 2008).

Pseudomonas aeruginosa (PA) is an opportunistic human pathogen that causes wide ranging infections in immunocompromised hosts and individuals with cystic fibrosis (Costerton *et al.,* 2001). It also causes bacteremia and wound infections and often form biofilm during infection (Fergie *et al.,* 1994; Harrison-Balistra *et al.,* 2003). The high degree of genomic flexibility and remarkable capacity to phenotypic adaptation of *P. aeruginosa* contributes to its ability for growth and persistence under various environmental and clinical settings (Wehmhoner *et al.,* 2003).

P. aeruginosa is considered as the best model pathogen to investigate biofilm production in clinical settings. (McDougald *et al.,* 2008). Various factors influence biofilm production by *P. aeruginosa* strains. These includes growth conditions, motility, cell surface properties, extracellular enzymes and various physicochemical stress factors, including various antibiotics (Mah *et al.,* 2008; O'Toole *et al.,* 2000). However, whether growth of bacteria in biofilm influences its potential to form new biofilm has not been investigated. This study was designed to investigate whether growth *P. aeruginosa* in biofilm has any influence on formation of new biofilm. Results obtained in this study show that in comparison to their isogenic planktonic counterparts, bacteria grown in biofilm has enhanced potential to form new biofilm.

2.Materials and Methods

Bacterial strains and biofilm assay: *P. aeruginosa* strains were obtained from King Khaled General Hospital, Hail, Saudi Arabia. Trypticase soy broth (TSB) and trypticase soy agar (TSA) plates were used to culture of bacteria as needed. Biofilm assay: Biofilm formation by *P. aeruginosa* strains was examined by crystal violet staining procedure (Moskowitz *et al.,* 2004). Overnight cultures of bacteria in TSB was diluted 1:100 in 3 ml of freshTSB contained in glass tubes and allowed to grow at 37 C in a static condition for 24 hours. Biofilms attached to the glass tubes were washed to remove inbound bacteria and stained with 1% (w/v) crystal violet for 10 min at room temperature. After washing with water, the stained biofilms were dissolved in 100% ethanol and the absorbance at 570 nm was determined

using a spectrophotometer and taken as a quantitative measure of biofilm production.

Table 1. Production of biofilm by clinical isolates of *P. aeruginosa*.

Strains	Clinical source	Biofilm production
B-6	Blood	0.92±0.28
B-9	Blood	0.64±0.22
W-2	Wound	1.20±0.33
W-14	Wound	1.02±0.23

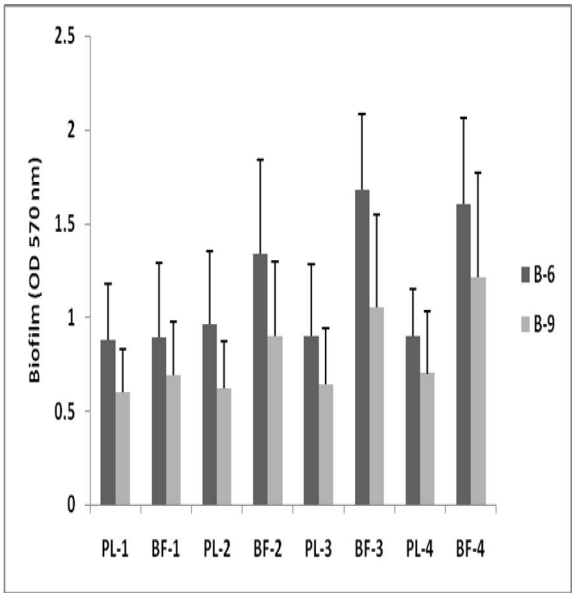

Figure 1. Production of biofilm by blood isolates of *P. aeruginosa* B-6 and B-9 at different passage levels (PL, planktonic; BF, biofilm). The results represent mean± standard deviations of three independent experiments.

Effect of passage on biofilm production: To explore the possibility whether passage in biofilm has any effect on new biofilm formation, the effect of passage of bacteria was systematically carried out. Biofilms were allowed to be formed by the *P. aeruginosa* strains in glass tubes containing TSB as described above, which was considered as the starter culture. After 24 hours of incubation at 37 C, 3 new glass tubes were inoculated with 10 ul of planknoic cells from the starter culture of each strain and 3 additional such TSB broth containing glass tubes were inoculated with one loop-full of bacteria taken for the formed biofilm of each starter culture. These tubes were marked as passage-1 (P-1). Planktonic cells and cells from biofilm of these P-1 cultures were used to generate P-2 cultures and so one. Biofilm was quantitated at each passage by crystal violet staining procedure as described above.

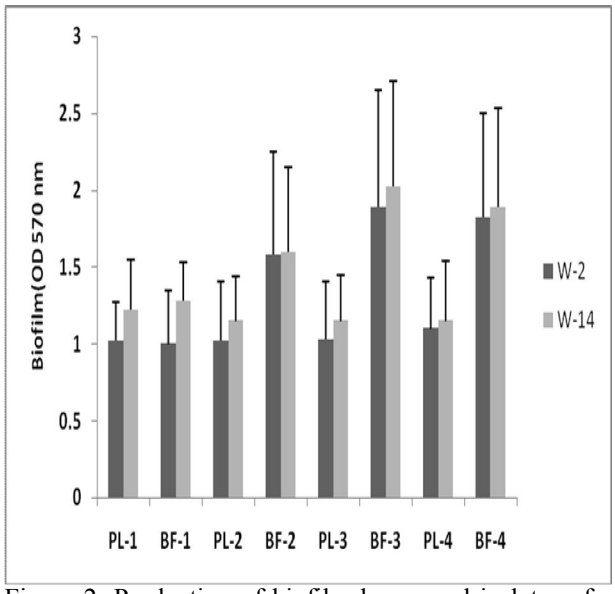

Figure 2. Production of biofilm by wound isolates of *P. aeruginosa* W-6 and W-14 at different passage levels (PL, passage in planktonic stage; BF, passage in biofilm stage). The results represents mean ± standard deviations of three independent experiments.

3.Results and Discussion

Formation of biofilm by bacteria is a complex process and studies directed to understand the biological process of biofilm formation have shown that a variety of environmental signals influence formation, maturation and dispersal of bacteria from formed biofilm to populate new sites (Parsek and Singh, 2003; Lopez *et al.,* 2010). While investigating the various factors that influence the production of biofilm by the *P. aeruginosa* strain B-6, it was observed that this strain produced higher amount of biofilm when TSB medium was inoculated with bacteria taken from biofilm in comparison to the same strain growing in planktonic form. This observation was then expanded with more strains and increasing the number of passages which is reported in this study.

Clinical origin and biofilm forming potential of the *P. aeruginosa* strains used in this study in presented in Table 1. All the strains produced biofilm following 24 hours of incubation at 37 C; however both the wound isolates produced relatively higher amounts of biofilm in comparison to the blood stream isolates. This finding is in agreement with previous studies which showed that wound isolates of *P. aeruginosa* were avid biofilm producer and biofilm could be detected within 10 hours of inoculation (Harrison-Balestra *et al.*, 2003). Figures 1 and 2 show the passage induced biofilm formation by the strains included in this study. Strains B-6, W-2 and W-14 showed gradual increase in biofilm formation in passages P-1, P-2, P-3. In

comparison to their planktonic counterparts, the biofilm grown bacteria showed 64.7, 83.4, and 75.6 % higher for the strains B-6, W-2 and W-14, respectively (Figures 1 and 2). These strains showed a partial decrease in biofim formation at P-4. On the other hand, strain B-9 showed continued increase in biofilm formation up to P-4. Biofilm production at passage 4 (P-4) by B-9 was 72.8% higher in comparison to the amount of biofilm produced by planktonic cells at passage P-4 (Figure-1). As 3 out of 4 strains showed maximal biofilm production at P-3 further passage was not carried out beyond P-4. These findings probably indicate that multiple levels of regulations are operative in biofilm formation and the level of modulation is varied in different strains. Studies have shown that two genetic loci of *P. aeruginosa, pel* and *psl* are involved in the production of extracellular material which serves as the encasing matrix of in biofilm (Friedman and Kolter, 2004). It would be interesting to investigate the modulation of expression of these two genes at different passage levels as different amounts of biofilm was produced by *P. aeruginosa* strains at different passage levels.

Table-2 Effect of Ferric Ammonium Citrate (FAC) on biofilm production by *P. aeruginosa* strains with and without passage.

Strain	FAC	Biofilm Production
B-6	----	1.02±0.22
B-6	250 µM	0.59±0.12
B-6/P-3	----	1.79±0.42
B-6/P-3	250 µM	1.60±0.37
W-14	----	1.29±0.31
W-14	250 µM	0.72±0.22
W-14/P-3	----	2.20±0.51
W-14/P-3	250 µM	1.99+0.45

Iron is required for growth of *P. aeruginosa* and iron has been shown to promote biofilm formation *in vitro*. Iron chelating agents such as EDTA and citrate salts have been found to exert a negative effect on the growth of the pathogen and these agents also exert an inhibitory effect on biofilm formation (Singh, 2004; Banin *et al.,* 2005). Ferric ammonium citrate (FAC) inhibited biofilm formation is a dose dependent manner (Banin *et al.,* 2005). In this study FAC was used as an biofilm inhibitory agent to determine whether the passage induced increased biofilm formation could abrogate the inhibitory effect of FAC. These results are presented in Table-2. Strain B-6/P-3 and W-14/P-3 are B-6 and W-14 strains at passage-3.FAC was used at 250 µM final concentration as initial experiments showed that its inhibitory effect on biofilm formation was maximum at this concentration (data not shown). Table-2 shows that the inhibitory effect of FAC on biofilm formation by the clinical strains of *P. aeruginosa* used in this could be counterbalanced in part by the serial passage of these strains in BF.

Studies have shown that Food and Drug Administration (FDA) approved iron chelators for human use such as deferoxamine and deferasirox could reduce formed biofilm and could partially inhibit formation of new biofilm formation on cystic fibrosis lung air way epithelial cells. The inhibitory effects of these iron chelators were further enhanced when these were used along with antibiotic and these being considered as a therapeutic option (Moreau-Marquis *et al.,* 2005). Considering the previously published results along with the finding of this study that passage in biofilm can override the inhibitory effect on biofilm formation it may be inferred that more ingenious drugs and therapeutic drugs need to be developed for successful treatment of chronic infections caused by *P. aeruginosa* in biofilm.

In this study it is demonstrated for the first time that passage in biofilm enhances the biofilm potential of new biofilm formation *P. aeruginosa* strains. Studies directed towards understanding the molecular basis of passage induced enhancement in biofilm production are in progress.

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