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Investigation of Antibacterial Property of Cockroach Wing Surface Against *Pseudomonas aeruginosa* Strains

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Abstract

Bacterial infection is a menace in the healthcare sector. Bacteria responsible for nosocomial infections have developed multi-drug resistance. This urges the need for adopting physical means to deter bacterial attachment. This study investigated the bactericidal activity of surface topography of cockroach wing against strains of Pseudomonas aeruginosa. The results indicated that the nanopore topography was found to reduce the bacterial attachment and their viability till 7h, suggesting that nanopore topography could be a potential antibacterial surface.

Keywords – Pseudomonas aeruginosa, nanopore, viability, bactericidal, cockroach

I. INTRODUCTION

Biomaterial implants are used to treat trauma, tumor resection or wear in the human body. Biomaterials replace or augment the functioning of the damaged organ either temporarily or permanently. Use of foreign body (biomaterial) inside the host system invites bacterial infections, which is termed as Biomaterial Associated Infection (BAI). Bacteria primarily attaches to the surface of the implant, multiplies and eventually forms a biofilm, leading to implant disfunctioning and demands revision surgery. Infection after total hip replacement occurred in 1.7% of patients after primary procedures and in 3.2% after revision procedure [1]. In the United States the overall infection burden from1990 to 2004 of hip arthroplasty was 0.88%, which showed annual increase at a rate of 5%. Conventional techniques employ antibiotics such as amoxicillin, cephalexin, gentamycin, sulfamethoxazole, ciprofloxacin and vancomycin. However, numerous bacterial species have developed multidrug-resistance [1].

Smart surfaces like Cu-bearing stainless steel surface, Ag releasing hydrogels, antibiotics releasing bone cements [10], and implant surface coated with N, N-dodecyl methyl-polyethylene imine were used to combat BAI. These smart surfaces with antibiotics have uncontrolled burst release, poor durability and long leaching time that leads to further complications [7].

Based on the reports of *Ivanova et al.2012*, natural surfaces are being exploited for their antimicrobial properties. Insects have evolved to possess super hydrophobic wing surfaces to minimize or prevent the attachment of contaminants on their surface. The super hydrophobicity also possesses self-cleaning properties. Insects follow different strategies to evade the attachment of contaminants. The bactericidal and antifouling nature of cicada and dragon fly wings are well studied [2].

The wing surfaces of cicada and dragonfly consists of nanopillars on the surface that contribute for its super hydrophobicity. These nanopillars were reported to rupture the cell walls of bacteria that come in contact [4, 8]. This bactericidal activity was reported to be due to the physical property of the nanopillars and not its chemical property [4].

This study aims to investigate the antibacterial property of the cockroach wings against *Pseudomonas aeruginosa* strains PAO1 and ATCC 9027.

II. MATERIALS AND METHODS

A. Sample preparation

Cockroaches were collected from the household areas in Madurai, India. The wings were rinsed with Millipore water (18.2 MΩ resistivity) and dried in laminar air flow chamber, followed by UV irradiation for 20 minutes. The wings were cut into circular discs of 1 cm diameter and stuck to 48-well plate (fig. 1). Glass coverslip was used as control [3].

Fig. 1: Glass cover slip and wings fixed to the bottom of a 48 well-plate.

B. Surface Characterization of Wing

1) Physical characterization

The cleaned wings were sputter coated with gold nanoparticles (Emitech Mini Sputter coater) and imaged under Scanning Electron microscope (ZEISS). Various regions of the wings were focused to confirm the morphological consistency.

2) Surface wettability

Static water contact angles were measured on cockroach wings using KRUSS-Drop Shape Analyzer DSA 25E. A volume of 10 µl of water was placed on the wing surface and the contact angles were measured.

3) Chemical Characterization

EDAX (Bruker) was used to determine the elemental composition of the wings. The elements present on the wing surfaces were detected from the spectra. The relative atomic percentage of the elements was determined.

C. Bacterial culture preparation

Pseudomonas aeruginosa strains PAO1 and ATCC 9027 (MTCC 1688) were procured from NCIL, Pune and MTCC, Chandigarh, India. Single colony of the cultures were inoculated into the nutrient broth and cultured overnight in a shaker incubator at 120 rpm. After incubation, the cultures were centrifuged at 7,000 rpm for 6 min to pellet down the cells. The pelleted cells were resuspended in PBS (pH 7.4) to reach OD_{600nm} to 0.1.

D. *Analysis of Bacterial viability on cockroach wing surface*

P. aeruginosa cultures suspended in PBS were loaded on to the wells of 48 well-plate containing a 1 cm diameter cockroach wings and glass cover slip. This plate was incubated at 37 ˚C. Readings were taken at discrete time intervals of 0.5 h, 1h, 2h, 7h, 24h and 48h. Post-incubation, the cultures were diluted 100 times with PBS pH 7.4. 10 µl of this diluted sample was used for plating on nutrient agar plates [3]. Meanwhile, to determine the number of viable cells attached to the substrate, the substratum was rinsed in PBS pH 7.4 and sonicated in 2 ml PBS pH7.4 for 10 min using a bath type sonicator (Ultra Sonic cleaner Lab Companion UCP-02). 10 µl from this sample was used for plating on nutrient agar plate. The plates were incubated over night at 37 ˚C. The colonies were counted and the relative colony forming units per ml were calculated using the formula below.

Colony forming units= (no. of colonies*dilution factor)/ Volume used for plating

E. Imaging of bacterial attachment

The surface topographies of the wings and bacterial attachment on the wings were visualized using SEM. The coverslips and wings obtained after incubation with P. aeruginosa strains were used for SEM imaging. The bacterial cells attached to the coverslip and wing were fixed with 2.5% (v/v) glutaraldehyde for 10 min. glutaraldehyde was discarded and the substrates were rinsed thrice with PBS. The samples were then dehydrated gradually using 30%, 50%, 70%, 90%, 100% and 100% (v/v) ethanol replacing each after every 10 minutes [5]. The samples were then air-dried. The dehydrated samples were then sputter coated with gold and mounted onto the studs for imaging in SEM.

F. DNA Quantification

The DNA in the suspension was quantified without disrupting the intact cells. This is used as an indirect measure of dead cells. The bacterial cultures suspended in PBS were incubated with coverslip and wing at 37ºC. After 30 min, 1h, 2h, 7h, 24h and 48h, the suspension from the 48 well-plate was collected and centrifuged to pellet down the cells. The DNA released into the supernatant was extracted by adding equal volume of mixture containing phenol:chloroform:isoamyl alcohol (25:24:1) and vortexed. It was then allowed to stand at room temperature for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 4˚C. Then the aqueous phase containing DNA was transferred to a clean 2 mL centrifuge tubes. To the aqueous phase, 1/10 volume of 3M sodium acetate and 2.5 volume of ice-cold absolute ethanol was added. Then the tubes were inverted to mix the contents and incubated overnight at -20˚C. After incubation, the tubes were centrifuged at 12,500 rpm for 15 minutes at 4ºC and supernatant was discarded. The pellet was washed with 70% ethanol. Then the pellets were resuspended in sterile water and absorbance was measured at 260 nm [9].

G. Estimation of EPS

In order to estimate the amount of extracellular polysaccharides (EPS) produced by both *Pseudomonas aeruginosa* PAO 1 and *Pseudomonas aeruginosa* ATCC 9027 on the wing surfaces of cockroach, the EPS produced on the substrate was extracted and then quantified according to the method previously method described [11].

After incubation of the *P. aeruginosa* cultures with coverslip/wing, the suspensions from the 48 well-plate were centrifuged at 12,500 rpm for 20 minutes at 4˚C. The supernatant was saved and to the pellet, 10 mM EDTA was added and vortexed for 15 minutes. Then the vortexed sample was centrifuged at 12500 rpm for 20 minutes at 4˚C. The supernatant was collected and pooled with the supernatant that was previously saved. The pooled sample was mixed with 2.2 volume of chilled absolute ethanol and incubated at -20˚C for 1 hour. Then the sample was again centrifuged at 12500 rpm for 20 minutes at 4˚C. The pellet containing the EPS was quantified by phenol – sulphuric acid method (*Dubois et al., 1956*). Briefly, to 1 ml of EPS extracted, a mixture containing 1 ml of 5% (v/v) phenol and 5 ml of sulphuric acid was added. Sulphuric acid was added rapidly, such that the stream of the acid was directed against the sides of the test tube. The mixture was vortexed and allowed to stand for 10 minutes, followed by incubation in water bath at 25-30 ˚C for 20 minutes. Then the absorbance of the samples was measured at 480 – 490 nm.

III. RESULTS AND DISCUSSION

A. Physical Characterization

1) Surface architecture

The surface topography of cockroach wing showed nano pore structures arranged in a flower like pattern (Fig. 2). The diameter of pores varied between 189.4 and 303.4 nm.

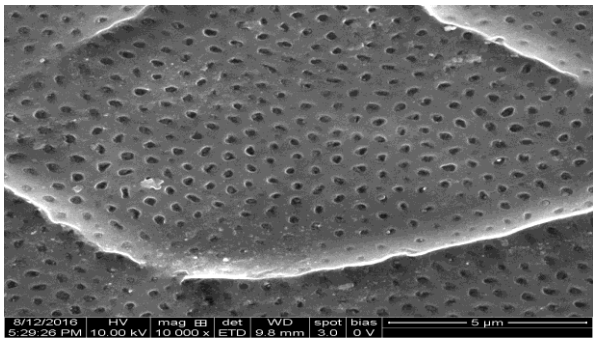


Fig. 2. Surface topography of cockroach wing surface

Scanning Electron Microscopic image cockroach wing surface at 10000 X magnification. Nano pore architecture with diameter varying between 189.4 and 303.4 nm was distributed throughout the wing surface.

2) Surface wettability

The surface hydrophobicity was tested using static water contact angle. Water contact angle on cockroach wing surface varied between 92˚ and 99˚ (fig. 3), indicating the hydrophobic nature of the surface.

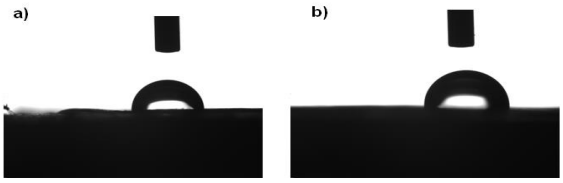
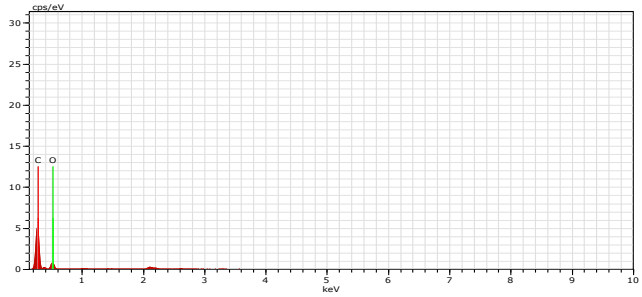


Fig. 3. Surface wettability analysis on cockroach wing surface. Water contact angle measurement on the wing surfaces ranged between (a) 92 degrees and (b) 99 degrees indicated the hydrophobicity of the surface.

B) Chemical Characterization

The EDX analysis allowed the determination of the elemental composition of cockroach wing (fig. 4). Previous studies [3,4] indicated that the wing surfaces were composed of hydrophobic long chain lipids.



El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
C	6	K-series	74.33	74.33	79.42	9.13
O	8	K-series	25.67	25.67	20.58	4.00
		Total:	100.00	100.00	100.00	

Fig 4. EDAX spectra of cockroach wing

C) Viability of P. aeruginosa on cockroach wing surface

The viable bacteria on the surface of coverslip and wing were estimated using colony counting technique. The viability results (fig. 5) indicated that *P. aeruginosa* PAO1 showed a decline in the viability from 30 min to 7 h ($8x10^5$ cfu/mL). After 7 h, the viable cell count was observed to increase both on coverslip and wing showing $40x10^5$ cfu/mL and $12x10^5$ cfu/mL at 48 h on coverslip and wing respectively.

P. aeruginosa ATCC 9027 also showed decline in viable cell count till 7 h ($200x10^5$ cfu/mL), after which the viable cells increased to $700x10^5$ cfu/mL on coverslip at 48 h. *P. aeruginosa* ATCC 9027 on wing surface showed decline till 7 h ($80x10^5$ cfu/mL), after which the viable cell count increased to $450x10^5$ cfu/mL.

The results indicated that the number of viable cells was lesser on wing than on the coverslip in both the strains, with PAO1 exhibiting lower viability on the wing surface. The viability pattern suggested that nanopore surface topography does exhibit bactericidal activity. Similar result was reported by Feng et al. 2015 when studied bacterial

viability on the aluminium surfaces with nanopore topography.

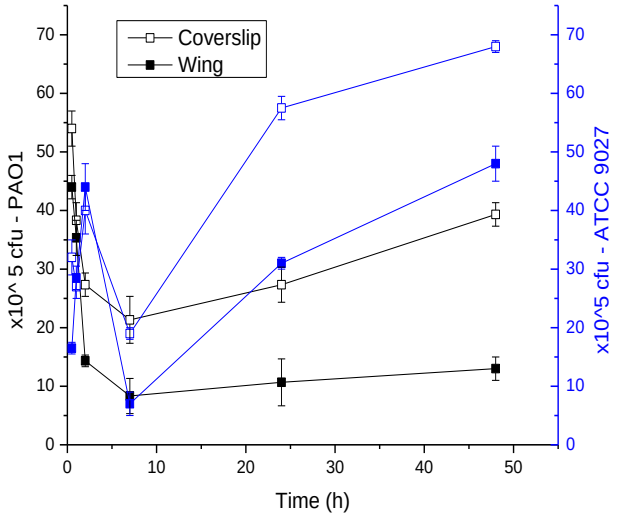


Fig. 5. Viability of *P. aeruginosa* strains on the coverslip and wing from 30 min to 48 h.

D) Imaging of Bacterial Attachment

P. aeruginosa strains attached to the wing surface after 7 h and 24 h of incubation were visualized and imaged under SEM (fig. 6). The images indicate that number of PAO1 attached to the wing surface was lesser than that of ATCC 9027 at tested time periods.

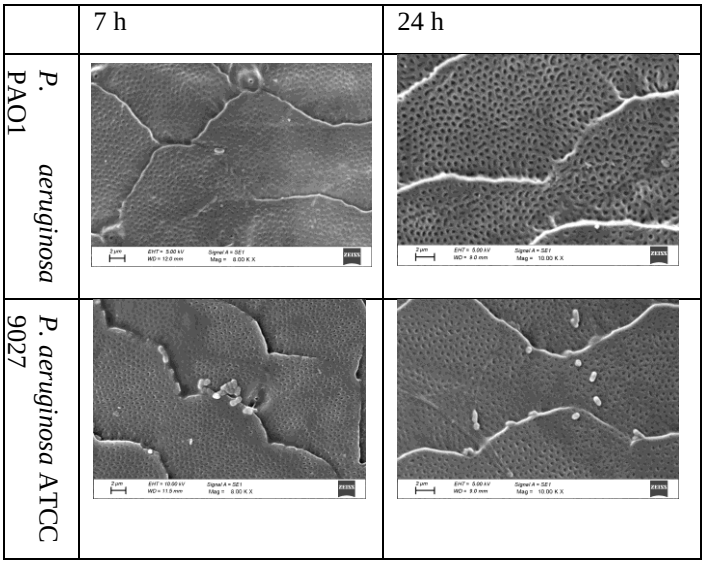


Fig. 6. SEM images of *P. aeruginosa* strains PAO1 and ATCC 9027 on the wing surface after 7 h and 24 h of incubation.

E. Quantification of DNA in supernatant

The DNA explicitly in the supernatant was precipitated and quantified without lysing the live intact cells. The results (fig. 7) indicated that the concentration of DNA in the supernatant was 10 µg/mL at 7 h when the *P. aeruginosa* strains were incubated with coverslip. This concentration increased to 12 µg/mL and 16 µg/mL in PAO1 and ATCC 9027 respectively at 48 h.

When *P. aeruginosa* strains were incubated with wings, the concentration of 2 µg/mL and 3.8 µg/mL at 7 h increased to 3.5 µg/mL and 4 µg/mL of DNA in PAO1 and ATCC 9027 respectively at 48 h. The results suggest that ATCC 9027 released more DNA than PAO1 on both coverslip and wing.

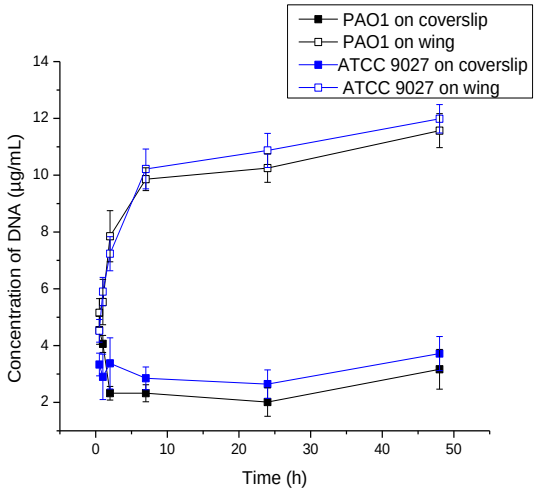


Fig. 7. Concentration of DNA in the supernatant when *P. aeruginosa* strains were incubated with coverslip and wing for 48 h.

F. Estimation of EPS

The EPS secreted by *P. aeruginosa* strains was estimated colorimetrically using phenol-sulphuric acid method. The results (fig. 8) indicated that the EPS secreted by PAO1 on coverslip increased from 38 µg/mL at 7h to 45 µg/mL at 48 h, while in ATCC 9027, the concentration of EPS increased from 22 µg/mL at 7 h to 30 µg/mL at 48 h. When the bacterial strains were incubated with wing surface, the strain PAO1 showed increase in EPS concentration from 53 µg/mL at 7 h to 40 µg/mL at 48 h and in ATCC 9027, the concentration of EPS increased from 28 µg/mL at 7 h to 40 µg/mL at 48 h. Though the concentration of EPS was higher in bacteria-wing interaction than bacterial interaction with coverslip, significant differences in the EPS concentration at 48 h was not observed.

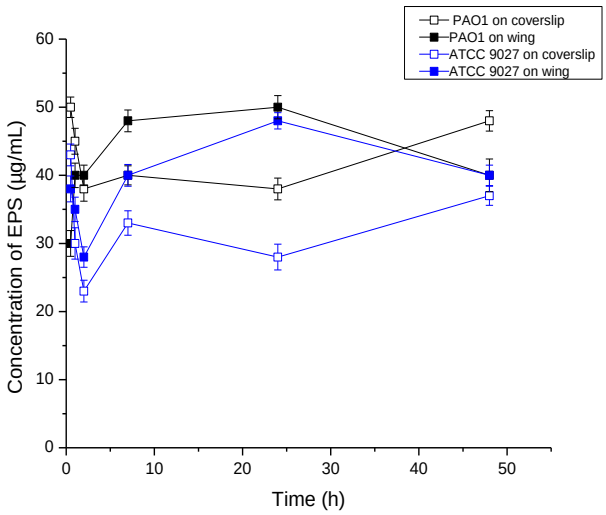


Fig. 8. Estimation of EPS secreted by *P. aeruginosa* strains PAO1 and ATCC 9027 when incubated with coverslip and wing for 48 h.

Feng *et al* 2014 reported significant decrease in the attachment of *Escherichia coli* ATCC 25922 and *Listeria innocua* when incubated with the titanium substrate having nanopores of 15 -25 nm. *E. coli* K12 strains were observed to have deterred in expressing the surface appendage when incubated with surface containing nanoscale pores and consequently inhibiting flagellar-medicated attachment.

IV. CONCLUSION

This study concludes that the nanopores on the wings of cockroach exhibited bactericidal activity against *P. aeruginosa* strains. Further experimentation on the dimensions of nanopores and material properties might facilitate in developing biomaterials with innovative and efficient surface architecture for better biological functionalities.

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REFERENCES

1) A. Bridier, R. Briandet, V. Thomas and F. Dubois-Brissonnet, "Resistance of bacterial biofilms to disinfectants: A Review", *Biofouling*, pp.1017–1032, 2011.
2) C. M. Bhadra, Vi Khanh Truong, Vy T. H. Pham, Mohammad Al Kobaisi, Gediminas Seniutinas, James Y. Wang, Saulius Juodkazis, Russell J. Crawford & Elena P. Ivanova, "Antibacterial titanium nanopatterned arrays inspired by dragonfly wings", *Sci. Rep.* 5:16817 (2015).
3) E. P. Ivanova, Jafar Hasan, Hayden K. Webb, Gediminas Gervinskas, Saulius Juodkazis, Vi Khanh Truong, Alex H.F. Wu, Robert N. Lamb, Vladimir A. Baulin, Gregory S. Watson, Jolanta A. Watson, David E. Mainwaring & Russell J. Crawford, "Bactericidal activity of black silicon", *Nat. Commun*. 4 : 2838 (2013).
4) E. P. Ivanova, Vi Khanh Truong, Hayden K. Webb, Vladimir A. Baulin, James Y. Wang, Narges Mohammodi, Feng Wang, Christopher Fluke & Russell J. Crawford, "Differential attraction and repulsion of *Staphylococcus aureus* and *Pseudomonas aeruginosa* on molecularly smooth titanium film". *Sci. Rep*. 1: 165 (2012).
5) G. Feng, Yifan Cheng, Shu-Yi Wang, Diana A Borca-Tasciuc, Randy W Worobo1 and Carmen I Moraru, "Bacterial attachment and biofilm formation on surfaces are reduced by small-diameter nanoscale pores: how small is small enough?" npj Biofilms and Microbiomes (2015).
6) G. Feng, Y. Cheng, Shu-Yi Wang, Lillian C. Hsu, Yazmin Feliz, Diana A. Borca-Tasciuc, Randy W. Worobo Carmen I. Moraru, "Alumina surfaces with nanoscale topography reduce attachment and biofilm formation by *Escherichia coli* and *Listeria spp*.," *Biofouling*, vol. 10, pp: 1253–1268 (2014).
7) J. Hasan, H. K. Webb, Vi Khanh Truong, Sergey Pogodin, Vladimir A. Baulin, Gregory S. Watson, Jolanta A. Watson, Russell J. Crawford & Elena P. Ivanova, "Selective bactericidal activity of nanopatterned superhydrophobic cicada *Psaltoda claripennis* wing surfaces". *Appl. Microbiol. Biotechnol.* (2012).
8) Kelleher S. M., Habimana O., Lawler J., B. O' Reilly, S. Daniels, E. Casey, and A. Cowley, "Cicada Wing Surface Topography: An Investigation into the Bactericidal Properties of Nanostructural Features". *ACS Appl. Mater.* Interfaces (2015).
9) M. C. Das, Padmani Sandhu, Priya Gupta, Prasenjit Rudrapaul, Utpal C. De, Prosun Tribedi, Yusuf Akhter & Surajit Bhattacharjee. (2016): "Attenuation of *Pseudomonas aeruginosa* biofilm formation by Vitexin: A combinatorial study with azithromycin and gentamicin". *Sci. Rep.* 6:23347 (2016).
10) M. Kostakioti, Maria Hadjifrangiskou, and Scott J. Hultgren, "Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Post antibiotic", Cold Spring Harb Perspect Med 3:a010306 (2013).
11) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, And Fred Smith, "Colorimetric Method for Determination of Sugars and Related Substances". *Anal. Chem.* (1956).