Biophysical model of bacterial cell interactions with nano-patterned cicada wing surfaces

Sergey Pogodin, † Jafar Hasan, ‡ Vladimir A. Baulin, ‡§ Hayden K. Webb, ‡ Vi Khanh Truong, ‡ The Hong Phong Nguyen, ‡ Veselin Boshkovikj, ‡ Christopher J. Fluke, ‡ Gregory S. Watson, ‡ Jolanta A. Watson, ‡ Russell J. Crawford ‡ and Elena P. Ivanova ‡

†Departament d'Enginyeria Quimica, Universitat Rovira i Virgili 26 Avenida dels Països Catalans, 43007 Tarragona, Spain.

‡Faculty Life and Social Sciences, and §Centre for Astrophysics and Supercomputing, Swinburne University of Technology, PO BOX 218, Hawthorn, Victoria, 3122, Australia

§ICREA, 23 Passeig Lluis Companys, 08010 Barcelona, Spain

‡Centre for Biodiscovery and Molecular Development of Therapeutics, School of Marine and Tropical Biology, James Cook University, Townsville, QLD, 4811, Australia

ABSTRACT

The nano-pattern on the surface of the Clanger cicada (Psaltoda claripennis) wings represents the first example of a new class of biomaterials that can kill bacteria on contact based solely on its physical surface structure. They provide a model for the development of novel functional surfaces that possess an increased resistance to bacterial contamination and infection. We propose a biophysical model of the interactions between bacterial cells and the cicada wing surface structures, and show that mechanical properties, in particular cell rigidity, are key factors in determining bacterial resistance/sensitivity to the bactericidal nature of the wing surface. This was experimentally confirmed by decreasing the rigidity of ‘surface-resistant’ strains through microwave irradiation of the cells, which renders them susceptible to the wing effects. These findings demonstrate the potential benefits of incorporating cicada wing nano-patterns into the design of antibacterial nanomaterials.

Keywords: cell/substratum interactions, cicada wings, nano-patterns, antibacterial nanomaterials

* Correspondence:

vladimir.baulin@urv.cat
eivanova@swin.edu.au
INTRODUCTION

Several surfaces exist in Nature that are capable of maintaining a contaminant-free status despite the innate abundance of potential contaminants in their surrounding environments (1-5). The vast majority of these surfaces owe their self-cleaning qualities to their superhydrophobic properties, which are in turn largely due to their physical surface structure. Many animals (e.g. sharks (6, 7), cicadae (8), butterflies (9), termites (10), mosquitos (11), geckos (12)) and plants (e.g. Lotus, Nelumbo nucifera (13, 14) and cabbage, Brassica oleracea (15)) possess hierarchical surface features that significantly increase their hydrophobicity, often to the point of becoming superhydrophobic (10, 16).

A number of research groups have attempted to establish a direct link between the self-cleaning and antibiofouling properties of surfaces, i.e. the ability to prevent attachment and accumulation of biological material (17-20). More recently, we have demonstrated that superhydrophobic/self-cleaning surfaces are not necessarily inherently antibiofouling in nature (8). Pseudomonas aeruginosa cells were found to be capable of adhering relatively effectively onto the surface of the wings of the Clanger cicada (Psaltoda claripennis); however those cells that were able to attach to the surface were killed with extreme efficiency by the wing surface (8). It was demonstrated that cicada wings were efficient against other Gram-negative bacteria, B. catarrhalis, E. coli and P. fluorescens, while Gram-positive bacteria B. subtilis, P. maritimus, and S. aureus remained resistant (21). This result suggested a common mechanism underlying the observed phenomenon. Even more significantly, it was also demonstrated that the bactericidal properties possessed by the wings took the form of mechanical rupture of the bacteria arising from the physical interactions between the cells and the nanoscale wing surface structure. These cicada wings were the first described example of a surface which possesses biocidal activity based solely on the physical surface structure (8).

The antibacterial properties of cicada wings have only very recently been discovered, and hence there is still much to be learned about the specific mechanisms that lead to the observed bactericidal behavior (8, 21). It is critical to obtain greater fundamental understanding of these mechanisms before any attempt can be made to apply these structures in medical contexts. A biophysical model was developed to provide an insight into the interactions taking place between the bacterial cells and the cicada wing surface structures. Adsorption of the bacterial cell membrane on the pattern of cicada wings surface may lead to a drastic increase of the total area, accompanied by the stretching of the membrane, which may in turn lead to irreversible membrane rupture and death of bacteria. Previously, gold coating of the cicada wings was shown to significantly alter the surface properties, while preserving both the topographical structure and subsequently the bactericidal effect (8). This observation led to two research hypotheses which are the focus of this work: (i) the mechanism is biophysical and no specific biological interactions play a role; and (ii) less rigid bacterial membranes will be more affected by the bactericidal mechanism of the wings.

MATERIALS AND METHODS

Sample preparation. Cicada (Psaltoda claripennis) specimens were collected from the greater Brisbane parkland areas. It is known that the cell regions of the dorsal and ventral sides of the wings possess a homogeneous nano-pattern on their surface (22). For consistency, all
experiments were performed on the same cell regions on the dorsal side of the forewing. Portions of the wings approximately 0.5 cm × 0.5 cm were excised by a scalpel or scissors and attached onto circular coverslips using adhesive tape. The wing samples were then briefly rinsed with MilliQ H₂O (resistivity of 18.2 MΩ cm⁻¹, Millipore, U.S.A.) and finally blow-dried using 99.99% purity nitrogen gas (23).

**Scanning electron microscopy.** High-resolution SEM images of cicada wings with adhering bacteria were taken using a field-emission SEM (FESEM; ZEISS SUPRA 40 VP, Germany) at 3 kV under 35000× and 42000× magnification. Samples were coated with thin gold films using a Dynavac CS300 before viewing with the microscope.

**Atomic force microscopy.** AFM scans were performed using an Innova microscope (Veeco, Bruker, U.S.A.) as described elsewhere (8). Briefly, scans were conducted using phosphorus doped silicon probes (MPP-31120-10, Veeco, Bruker) with a spring constant of 0.9 N/m, tip radius of curvature of 8 nm and a resonance frequency of ~20 kHz were utilized for surface imaging. Scanning was carried out in tapping mode perpendicular to the axis of the cantilever at 1 Hz.

**Bacterial strains, growth and sample preparation.** *Bacillus subtilis* NCIMB 3610ᵀ, *Planococcus maritimus* KMM 3738 and *Staphylococcus aureus* CIP 65.8ᵀ were used in this study. Bacterial strains were obtained from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, UK), the Collection of Marine Bacteria (KMM, Russian Federation) and the Culture Collection of the Institute Pasteur (CIP, France). Prior to each experiment, bacterial cultures were refreshed from stocks on nutrient agar (Oxoid, U.K.) or marine agar (BD, U.S.A.). For cell attachment experiments, fresh bacterial suspensions were prepared for each strain grown overnight at 37 °C in 5 mL of nutrient broth (Oxoid) or at 25 ºC in 5 mL of marine broth (Difco) with shaking (120 rpm). Bacterial cells were collected at the logarithmic stage of growth, and the suspensions adjusted to OD₆₀₀ = 0.3, as described elsewhere (8). The mounted insect wings were immersed in 5 mL of the bacterial suspension, and incubated for 18 hours.

**Confocal laser scanning microscopy (CLSM).** Live and dead bacterial cells were visualized and differentiated using a Fluoview FV10i inverted CLSM system (Olympus, Tokyo, Japan). Cells were stained using the LIVE/DEAD® BacLight™ Bacterial Viability Kit, L7012, which contains a mixture of SYTO® 9 and propidium iodide fluorescent dyes (Molecular Probes™, Invitrogen, NY, USA) according to the manufacturer’s protocol. SYTO® 9 permeates all cells, binding to DNA, causing a green fluorescence. Propidium iodide only enters cells that have significant membrane damage, which is an indication of non-viability, and binds to nucleic acids with higher affinity than SYTO® 9.

**Microwave experiments.** Bacterial samples for MW treatment were comprised of 2 mL of cell suspensions (OD₆₀₀ = 0.1) that were transferred into a micro Petri dish 35 mm i.d. (Greiner Bio-One, Frickenhausen, Germany). The MW apparatus used was a Lambda Technologies Vari-Wave Model LT 1500, with the frequency fixed at 18 GHz and other settings as described elsewhere (24). The bulk temperature of the bacterial suspension during exposure was controlled to remain below 40 ºC at all times. Each sample was exposed to MW radiation for three consecutive exposures of one minute each, allowing the sample to cool back down to 20 ºC
between exposures. After treatment, the cell suspensions were incubated on insect wings mounted on circular coverslips, in the same manner as the untreated cells.

RESULTS AND DISCUSSION
The surface structure of the wings of *Psaltoda claripennis* has been extensively characterized by AFM and SEM imaging techniques and described in earlier reports (8, 21-23). It is well documented and was confirmed that the wing surfaces were covered by an array of nanopillar structures, arranged approximately hexagonally, spaced 170 nm apart from center to center (Figure 1). Each pillar was approximately 200 nm tall, of conical shape, with a spherical cap 60 nm in diameter.

*Pseudomonas aeruginosa* ATCC 9027 bacterial cells in contact with cicada wings are known to be deformed and mechanically ruptured by the nano-pattern on the surface of the wing (8). As the characteristic dimensions of the nanopillars on the surface of the cicada wings (∼100 nm) are an order of magnitude larger than the thickness of the bacterial membrane (∼10 nm) (25), the membrane can therefore be modeled as a thin elastic layer and the details of the structure and composition of the layer can be neglected. Similarly, as the typical size of a bacterial cell (i.e. ∼500 – 1000 nm) is at least several times larger than the spacing between the nanopillars, the curvature of the bacterial surface can also be ignored in the first approximation, and we can limit the consideration to the adsorption of a planar piece of a membrane onto array of nanopillars. In our model, the increase of the total area due to adsorption on the pillars leads to non-uniform stretching due to specific pattern, which leads in turn to membrane rupture.

In such a macroscopic description, the bacterial outer layer is characterized by the stretching modulus ($k$), the surface density of the attraction sites on the relaxed layer ($n_0$), and the energy gain per adsorption site ($\varepsilon$). The microscopic nature of the attraction forces between the layer and the nanopillars is absorbed into a single parameter $\varepsilon$, thus providing a certain degree of universality. The stretching of the layer due to the adsorption is described by the local relative stretching degree $\alpha(r)$ at point $r$. Assuming that the unperturbed membrane is characterized by the total area $S_0$, the initial stretching $\alpha_i$ and initial uniform density $n_0$ of the adsorption sites. The stretching due to contact with pillars on the surface of cicada wings leads to the redistribution of the adsorption sites from $n_0$ to the local density $n(r) = n_0/(1 + \alpha(r))$. Each site adsorbed on the nanopillar surface contributes the energy gain $\varepsilon \cdot \varepsilon$; therefore the total free energy gain due to the adsorption is given by:

$$ F^{\text{gain}} = \int_A \varepsilon n(r) d\sigma = \int_A \frac{\varepsilon n_0 d\sigma}{1 + \alpha(r)} \quad (1) $$

where $d\sigma$ is an element of the layer surface area, and the integration is performed over the total contact area $A$ between the layer and nanopillars surface ($A$).

Energy gain due to adsorption on the nanopillars is balanced by the free energy loss associated with the deformation of the membrane. The main contribution to the energy loss, $F^{\text{loss}}$, is due to local membrane stretching/compression, which is proportional to $(k/2)\alpha^2(r)$ for weak local deformations, $|\alpha(r)| \ll 1$. Thus, the integration over the total adsorbed area of the layer ($A$) plus the total area of the layer suspended between the nanopillars ($B$) gives

$$ F^{\text{loss}} = \int_{A+B} \frac{k}{2} \alpha^2(r) \frac{n(r)}{n_0} d\sigma = \int_{A+B} \frac{k}{2} \alpha^2(r) \frac{d\sigma}{1 + \alpha(r)} \quad (2) $$
The local stretching $\alpha(r)$ is not completely independent variable. It relates unperturbed area prior to adsorption and the area stretched due to adsorption through the following geometrical condition: the projection of unperturbed and stretched areas on the pillars plane remains constant. This condition can be taken into account in the total free energy with the help of Lagrange multiplier $\lambda$:

$$F = F^{\text{gain}} + F^{\text{loss}} + \lambda k \left( \int_{\rho_{A,B}} \frac{d\sigma}{1 + \alpha(r)} - S_0 \right), \quad (3)$$

where $S_0$ is the unperturbed area. Minimization of this expression with respect to $\alpha(r)$ yields the local stretching of the layer in the region $A$, where the membrane interacts with the nanopillars, and the region $B$, where the membrane is suspended between pillars, leading to the following condition:

$$1 + \alpha(r) = \begin{cases} 1 + \alpha_A = \sqrt{1 + 2(\lambda - \zeta)}, & \text{region A} \\ 1 + \alpha_B = \sqrt{1 + 2\lambda}, & \text{region B} \end{cases} \quad (4)$$

where the dimensionless effective interaction parameter $\zeta = -\epsilon n_0/k$ is defined as the ratio between the attraction of the layer to the nanopillars surface and the layer elasticity. Equations 4 lead to an important general conclusion. In the case where adsorption $\epsilon$ is negative and hence $\zeta$ is positive, the stretching of the suspended region of the layer, $\alpha_B$ is higher than the stretching of the adsorbed region of the layer, $\alpha_A$. This means that the rupture point of the layer will always be reached first in region $B$. In other words, the nanopillars do not pierce the membrane, but rather break the membrane between the nanopillars. One might imagine a scenario in which the nanopillars pierce the layer like an array of needles, however this would only be the case if the diameter of the spherical caps were much smaller than is actually the case, e.g. $\approx 1$ nm as opposed to the measured 60 nm.

Since $\alpha_A$ and $\alpha_B$ are constant for all points inside the regions $A$ and $B$ respectively, Eq. 3 can be simplified by converting the integrals into the areas $S_A$ and $S_B$ of the corresponding regions of the layer:

$$F = \frac{en_0 S_A}{1 + \alpha_A} + \frac{k}{2} \left( \frac{\alpha_A^2 S_A}{1 + \alpha_A} + \frac{\alpha_B^2 S_B}{1 + \alpha_B} \right) + \lambda k \left( \frac{S_A}{1 + \alpha_A} + \frac{S_B}{1 + \alpha_B} - \frac{S_i}{1 + \alpha_i} \right). \quad (5)$$

Here we consider that the unperturbed membrane is stretched up to the initial stretching degree $\alpha_i$ and the total initial area of unperturbed membrane is $S_i$.

The geometry of the nano-pattern on the surface of cicada wings is described by four parameters (Figure 2a): the radius of the cap on the top of the pillar $R = 30$ nm, the pillar height $h = 200$ nm, the pillar pitch $\beta = 10^\circ$, and the average distance between the pillars $d = 170$ nm. Assuming that the membrane suspended between the pillars (region $B$) remains horizontal with respect to the plane of the wing, the membrane position can be characterized by a single parameter, the vertical distance $x$ from region $B$ to the tip of the nanopillars. It is also convenient to consider separately two cases: the region $B$ is above (case I) and the region $B$ is below (case II) the junction point $M$ between the spherical cap and conical column of the nanopillar (Figure 2a). In case I it is more convenient to describe the position of the layer by the angle $\theta$ between the nanopillar vertical axis and the contact point between the nanopillar and region $B$ of the layer. In case II the most convenient parameter is the vertical distance $z$ between region $B$ and the junction point $M$. 

Assuming, that the average initial area of the layer per nanopillar is \( S_i = d^2 \), the areas \( S_A \) and \( S_B \) are given by the following expressions:

\[
S_A = 2 \pi R^2 \left( 1 - \cos \theta \right) \\
S_B = d^2 - \pi R^2 \sin^2 \theta \\
S_A = 2 \pi R^2 \left( 1 - \sin \beta \right) + \frac{2 \pi \rho}{\cos \beta} \left( R \cos \beta + \frac{z}{2} \tan \beta \right) \\
S_B = d^2 - \pi \left( R \cos \beta + \frac{z}{2} \tan \beta \right)^2
\]

These expressions allow for numerical minimization of the free energy, which gives the equilibrium position and the equilibrium stretching of the membrane. Figure 2b shows the calculated dependencies of the membrane stretchings \( \alpha_A \) (region A) and \( \alpha_B \) (region B) on the effective interaction parameter \( \zeta \) for different values of the initial degree of stretching \( \alpha_i \). It was found that \( \alpha_B \) increases continuously as \( \zeta \) increases. This suggests that there is a critical value \( \zeta_{\text{critical}} \) of the layer parameter \( \zeta \), at which \( \alpha_B \) also reaches a critical value and the membrane is ruptured.

The model suggests that the bactericidal mechanism is biophysical and does not imply directly any specific biological interactions. This is consistent with a previous experiment in which cicada wing surfaces were coated with gold (8). This technique allows preserving the geometry of the wings, but changing of the surface properties. The result demonstrates that such a pattern is lethal for \( P. \) aeruginosa cells, despite the substantial difference in surface chemistry. In order to explore the predictions of the proposed model and check the universality of the mechanism, we investigated the attachment behavior of two species of Gram-positive cocci, \( \text{Planococcus maritimus} \) and \( \text{S. aureus} \), and the Gram-positive rod-shaped bacterium \( \text{Bacillus subtilis} \) on cicada wing surfaces (21). It is well documented that Gram-positive bacteria are generally more rigid than their rod-shaped counterparts mostly due to the larger proportion of peptidoglycan present in the cell wall (25-27). We therefore performed comparative attachment experiments to determine whether Gram-positive cells respond in a similar manner to the Gram-negative \( \text{Pseudomonas aeruginosa} \). The results of this experiment revealed that all three species were unaffected by the nanopillar structures on the wing surface (Figure 3). Scanning electron micrographs showed clearly that the cells retained their characteristic morphologies, and confocal laser scanning microscopy confirmed that cells remained viable. According to the model, effective interaction parameter \( \zeta \) is proportional to the attraction between the bacterial layer and the wing surface, and is inversely proportional to the layer rigidity. Thus more rigid cells require stronger interaction with the surface in order to sufficiently stretch to the point of rupture. This offers a possible explanation for the resistance of \( B. \) subtilis, \( \text{Planococcus maritimus} \) and \( \text{S. aureus} \) to the action of the cicada wings, in that they possess increased rigidity relative to \( \text{Pseudomonas aeruginosa} \) (25-27).

If the presented model stands, then it would be expected that if the cell rigidity is decreased and/or the initial stretching on the membrane is sufficiently decreased, the bacterial strains that were previously resistant to the bactericidal action of the wing surface could potentially become susceptible. Microwave (MW) exposures under specified conditions have previously been shown to induce reversible poration in the membranes of bacteria (28-29), allowing the release of some of the cellular contents, decreasing internal turgor pressure and releasing some of the tension on the membrane. However, this technique itself is not lethal to the cells and the pores in the
membranes are self-sealed after few minutes. To test our conclusions, cells of *B. subtilis*, *Planococcus maritimus* and *S. aureus* were each exposed to microwave radiation, and then incubated in the presence of cicada wings. The morphology of irradiated cells that came into contact with the wing surface was markedly different to that observed for the non-irradiated cells (Figure 3B). The MW-treated cells were considerably deformed by the nanopillars, in a similar manner to that of the untreated *Pseudomonas aeruginosa* (8), confirming that decrease of the turgor pressure induced by the microwave treatment had rendered these cells susceptible to the bactericidal action of the wing surface. Subsequent viability experiments confirmed that the cells were indeed inactivated (Figure 3B). This is compelling evidence in support of the proposed model, confirming that the primary factors that determine the vulnerability of bacteria to the action of the wing surface are mechanical properties of the membranes: the rigidity and initial stretching.

**CONCLUSION**

A biophysical model of interaction of bacterial cells with superhydrophobic nanopillar structures on the surface of cicada wings was developed to provide increased fundamental understanding of the mechanisms behind the recently discovered phenomenon of the bactericidal action of cicada wings. As the bacterial cells adsorb onto the nanopillar structures present on the wing surfaces, the cell membrane stretches in the regions suspended between the pillars. If the degree of stretching is sufficient, this will lead to cell rupture. Gram-positive cells have a greater natural resistance to this effect than do Gram-negative cells, due to their greater rigidity. However by decreasing their internal turgor pressure and hence initial stretching and to some degree rigidity, through microwave irradiation these cells can be rendered sensitive to the bactericidal mechanisms of the wing surfaces. Designing bio/nanomaterials that possess cicada wing-like structures may be a promising avenue of research for applications in which minimizing bacterial contamination/infection is desirable.

**GRANTS**

This research was funded in part by the Advanced Manufacturing Co-operative Research Centre (AMCRC).

**REFERENCES**


FIGURE CAPTIONS

Figure 1. Cicada *Psaltoda claripennis* wing surface topography. a, Scanning electron micrograph of the surface of a cicada wing as viewed from above (Scale bar = 200 nm). b, Three-dimensional representation of the surface architecture of a cicada wing, constructed from AFM scan data and colored according to height. A three-dimensional animation of the cicada wing surface is available in the supplementary material and at [http://youtu.be/JDOEAUdqJGk](http://youtu.be/JDOEAUdqJGk).
Figure 2. Biophysical model of the cicada *Psaltoda claripennis* wing nanopillar – bacterial cell interactions. **a**, Schematic of a bacterial outer layer adsorbing on cicada wing nanopillars. The adsorbed layer can be divided into two regions: region (A), in contact with the pillars, and region (B), suspended between the pillars. As region A adsorbs and the surface area of the region ($S_A$)
increases, region B is stretched and eventually ruptures. **b-e**, Three dimensional representation of the modeled interactions between a rod-shaped cell and the wing surface. As the cell comes into contact (b) and adsorbs onto the nanopillars (c), the outer layer begins to rupture in the regions between the pillars (d) and collapses onto the surface (e). Images **b-e** are screenshots from an animation of the mechanism available at [http://youtu.be/KSdMYX4gqp8](http://youtu.be/KSdMYX4gqp8).

---

**Figure 3.** Modeled stretching dynamics of the outer layer of a bacterial cell in contact with a cicada wing surface. **a**, Stretching in the region A ($\alpha_A$, dash lines) and stretching in the region B ($\alpha_B$, solid lines) plotted as a function of the layer parameter $\zeta$, for layers under different degrees of initial stretching ($\alpha_i$), denoted by color. **b**, Stretching $\alpha_A$ and $\alpha_B$ plotted as functions of the position of the layer relative to junction point M between the spherical cap and conical base of
the nanopillars. Both $\alpha_A$ and $\alpha_B$ are plotted for different combinations of $\zeta$ and $\alpha_i$. The equilibrium position of the layer in each case is marked with dot.

**Figure 4.** Cell interactions of surface-resistant *B. subtilis* NCIMB 3610$^T$, *Planococcus maritimus* KMM 3738 and *S. aureus* CIP 65.8$^T$ strains after microwave (MW) irradiation. All three strains were rendered susceptible to the action of the wing surface by MW treatment. Typical scanning electron micrographs (left) show substantial deformation of the cell morphologies of all three species. Confocal laser-scanning microscopy viability analysis (right) demonstrated that all cells were inactivated (shown in red).