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ENVIRONMENTAL BIOTECHNOLOGY

Selective bactericidal activity of nanopatterned superhydrophobic cicada *Psaltoda claripennis* wing surfaces

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Abstract The nanopattern on the surface of 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. As such, they provide a model for the development of novel functional surfaces that possess an increased resistance to bacterial contamination and infection. Their effectiveness against a wide spectrum of bacteria, however, is yet to be established. Here, the bactericidal properties of the wings were tested against several bacterial species, possessing a range of combinations of morphology and cell wall type. The tested species were primarily pathogens, and included *Bacillus subtilis*, *Branhamella catarrhalis*, *Escherichia coli*, *Planococcus maritimus*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Staphylococcus aureus*. The wings were found to consistently kill Gram-negative cells (i.e., *B. catarrhalis*, *E. coli*, *P. aeruginosa*, and *P. fluorescens*), while Gram-positive cells (*B. subtilis*, *P. maritimus*, and *S. aureus*) remained resistant. The morphology of the cells did not appear to play any role in determining cell susceptibility. The bactericidal activity of the wing was also found to be quite efficient; $6.1 \pm 1.5 \times 10^6$ *P. aeruginosa* cells in suspension were inactivated per square centimeter of wing surface after 30-min incubation. These findings demonstrate the potential for the development of selective bactericidal surfaces incorporating cicada wing nanopatterns into the design.

Keywords Self-cleaning · Nanopattern · Bactericidal · Insect wings · Antibiofouling

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Introduction

Several naturally existing surfaces such as insect wings and plant leaves are capable of maintaining a contaminant-free status despite the innate abundance of potential contaminants in their surrounding environments (Bhushan and Jung 2011; Guo et al. 2011; Marmur 2004; Su et al. 2010; Webb et al. 2011). These self-cleaning properties arise from extreme degrees of hydrophobicity; water droplets repelled by the surface sweep away potential contaminating particles. We have recently reported that the wings of the Clanger cicada (*Psaltoda claripennis*) not only possess self-cleaning ability but are also antibacterial in nature (Ivanova et al. 2012). It was demonstrated that *Pseudomonas aeruginosa* cells that settled on the wing were ruptured with extreme efficiency by the nanopillar array on the wing surface. The newly discovered antibacterial properties of cicada wings have extensive potential in the field of bactericidal surfaces design. Postoperative infections and antibiotic resistance continue to be significant health concerns (Harbarth et al. 2002; Harris et al. 2010; Perez

et al. 2007; Rubin et al. 1999; Stamm 2010); however, surfaces that possess cicada wing-like topographies may eliminate pathogenic bacteria without the need for drug-based treatments. It is thought that cicada wing nanopillar patterns may be suitable for use in vivo (Green et al. 2012), such as on the surfaces of medical implants; however the effectiveness of such topographies against a range of pathogenic bacteria first needs to be established.

The aim of this work was to investigate the antibacterial effect of Clanger cicada (*P. claripennis*) wing surfaces against a range of bacteria with different combinations of morphology (rod-shaped vs coccus) and cell wall type (Gram-positive vs Gram-negative bacteria) including *Bacillus subtilis* National Collection of Industrial, Food and Marine Bacteria (NCIMB) 3610^T, *Branhamella catarrhalis* American Type Culture Collection (ATCC) 23246, *Escherichia coli* K12, *Planococcus maritimus* Collection of Marine Microorganisms (KMM) 3738, *P. aeruginosa* ATCC 9027, *Pseudomonas fluorescens* ATCC 49642, and *Staphylococcus aureus* Culture Collection of the Institut Pasteur (CIP) 65.8^T; and evaluate the efficiency of the observed bactericidal effect.

Materials and methods

Insect wings samples

Cicada (*P. claripennis*) specimens were collected from the greater Brisbane parkland areas. The wing samples were prepared as described elsewhere (Ivanova et al. 2012).

Bacterial strains and growth conditions

B. subtilis NCIMB 3610^T, *B. catarrhalis* ATCC 23246, *E. coli* K12, *P. maritimus* KMM 3738, *P. aeruginosa* ATCC 9027, *P. fluorescens* ATCC 49642. and *S. aureus* CIP 65.8^T were used in this study. Bacterial strains were obtained from the NCIMB (Aberdeen, UK), ATCC (Manassas, VA, USA), CIP (Paris, France), and the KMM (Vladivostok, Russian Federation).

Prior to each experiment, bacterial cultures were refreshed from stocks on nutrient agar (Oxoid, Basingstoke, Hampshire, UK) or marine agar (BD, Franklin Lakes, NJ, USA). 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 °C in 5 mL of marine broth (BD) with shaking (120 rpm). Bacterial cells were collected at the logarithmic stage of growth, and the suspensions adjusted to OD_{600}=0.3, as described elsewhere (Truong et al. 2010). The mounted insect wings were immersed in 5 mL of the bacterial suspension and incubated for 18 h. The controls for these experiments consisted of the same bacterial suspensions incubated on the glass cover slips.

Bacterial cell visualization

Before viewing, wing samples with adsorbed bacteria were first coated with thin gold films using a Dynavac CS300 according to the procedure previously developed (Mitik-Dineva et al. 2009; Truong et al. 2009). A field-emission SEM (ZEISS SUPRA 40 VP, Oberkochen, BW, Germany) set at 3 kV was used to obtain high-resolution electron micrographs of cicada wings with adhering bacteria at×15,000 and×35,000 magnification. Live and dead bacterial cells were visualized and differentiated using a FluoView FV10i inverted confocal laser scanning microscopy (CLSM) system (Olympus, Tokyo, Japan). Cells were stained using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit, L7012, which contains a mixture of SYTO® 9 and propidium iodide fluorescent dyes (Molecular Probes™, Invitrogen, Grand Island, 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 nonviability and binds to nucleic acids with higher affinity than SYTO® 9.

Bactericidal efficiency of cicada wings

P. aeruginosa cells were grown in 20 mL of nutrient broth for 18 h before harvesting, were resuspended in 5 mL of phosphate buffered saline, 10 mM, pH 7.4, and adjusted to OD_{600}=0.1 using a spectrophotometer (Dynamica Halo RB-10 UV–vis, Precisa Gravimetrics AG, Dietikon, Switzerland). Resuspended cells were diluted 1:10; then incubated in 3.5-cm diameter wells in triplicate, each well containing a 0.25-cm^2 piece of cicada wing. Five control wells contained only bacterial suspensions. The cell suspensions were then sampled (100 μL) at discrete time intervals, i.e., 1, 10, and 30 min; serially diluted three times by a factor of 10; and 10 μL of each dilution were spread on ten nutrient agar plates. Viability assays were performed using direct plate counting technique (Postgate 1969). The number of colony forming units was assumed to be equivalent to the number of live cells in suspension. All statistical data processing were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) as described elsewhere (Shamis et al. 2009).

Results

Gram-negative bacterial interactions with cicada wing surfaces

The *B. catarrhalis*, *E. coli*, *P. aeruginosa*, and *P. fluorescens* cells that attached to the surface of the wing all exhibited irregular morphologies (Fig. 1). The morphology of each of these cells resembled that observed for *P. aeruginosa* in the first

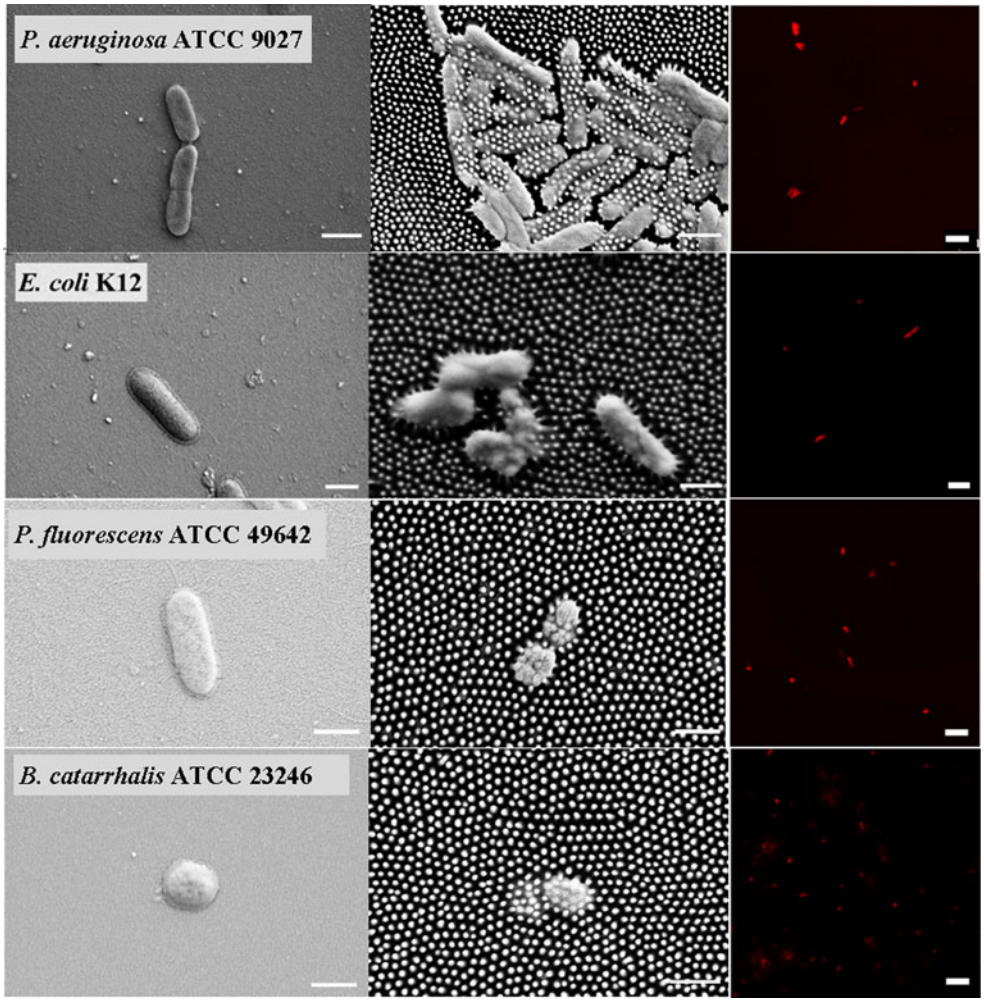

Fig. 1 Bactericidal activity of the cicada wing surfaces. Cells were incubated on wings (*center*) and glass (*left*) to identify morphological changes induced by the wing surface as observed by scanning micrograph images. Confocal laser scanning microscopy was used to determine viability postincubation; viable cells were labeled *green* and nonviable cells *red* in the CLSM images (*right*). Results indicated that the wing surface was lethal to all Gram-negative cells, regardless of morphology. *Scale bars* in electron micrographs=1 μm; in CLSM images, *scale bars*=5 μm

reported case describing the bactericidal activity of cicada wings (Ivanova et al. 2012). In that work, the cells were found to "sink" onto the surface, which was covered in an array of spherically capped, conical, nanoscale pillars. This caused substantial deformation of the cells and was shown to be lethal. Similarly, *B. catarrhalis*, *E. coli*, *P aeruginosa*, and *P. fluorescens* were all shown, through viability experiments, to be inactivated by the wing surface. Every cell of these four species that was visible appeared red in fluorescence micrographs (Fig. 1), indicating the binding of the propidium iodide fluorescent stain, and that these cells were dead. These results suggest that Gram-negative bacteria, irrespective of whether they are coccoid-shaped or rod-shaped, are substantially deformed and ultimately killed by the surface of *P. claripennis* wings.

Gram-positive bacterial interactions with cicada wing surfaces

In addition to the four Gram-negative species tested on the wing surface, the interactions between the cicada wings and three Gram-positive species were also analyzed. The Gram-positive, rod-shaped *B. subtilis* and the coccus *S. aureus*, as well as the Gram-variable coccus *P. maritimus* were tested to ensure that every combination of cellular morphology and cell wall structure was tested. The morphologies of *B. subtilis*, *S. aureus*, and *P. maritimus* on the cicada wing surface were effectively unchanged in comparison to that of cells attached to the glass surface controls (Fig. 2). To confirm that the wing surface was ineffective against these species, viability analysis was performed via CLSM. Every cell of all three species appeared green (Fig. 2), which indicated that only SYTO® 9 was able to stain the cells, and that they were still viable. In the cases of both the Gram-negative and the Gram-positive cells, cellular inactivation by the wing occurred irrespective of morphology. The only factor which was found to play a role in determining the susceptibility of the cells was the cell wall structure; Gram-negative cells were all found to be sensitive to the wing surface, while all Gram-positive cells were resistant.

Efficiency of cicada wing surfaces bactericidal activity

The kinetics of the bactericidal activity of the cicada wings was tested by standard viability plate counts using *P.*

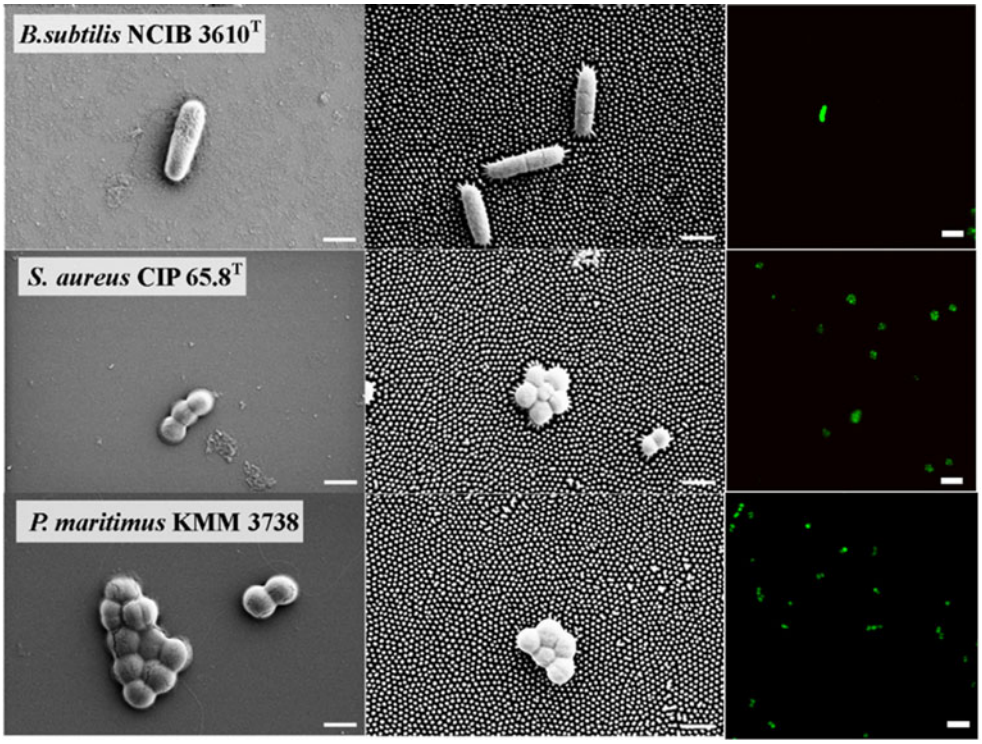

Fig. 2 Cell/surface interactions between Gram-positive cells and cicada wing surfaces. Gram-positive cells were incubated on wings (*center*) and glass (*left*) to identify any morphological changes induced by the wing surface. Confocal laser scanning microscopy was used to determine viability postincubation; viable cells were labeled *green* and nonviable cells *red* in the CLSM images (*right*). Results indicated that the wing surface had little to no effect on either the morphology or viability of Gram-positive cells. *Scale bars* in electron micrographs=1 μm; in CLSM images, *scale bars*=5 μm

aeruginosa, the organism against which the bactericidal activity was first reported. Over a 30-min incubation period in the presence of the cicada wings, the *P. aeruginosa* cells inactivated by the surface reached $6.14 \pm 1.50 \times 10^6$ cfu cm^{-2} (Fig. 3). The FDA requirements for testing antiseptics and disinfectants against bacteria recommend the used of relatively small inocula concentrations; approximately 1,000 cfu mL^{-1} (FDA 2001, 2009). The FDA guidelines, however, make reference to the bactericidal action throughout bulk food samples, whereas in this study, we are only concerned with the bactericidal ability of a material surface.

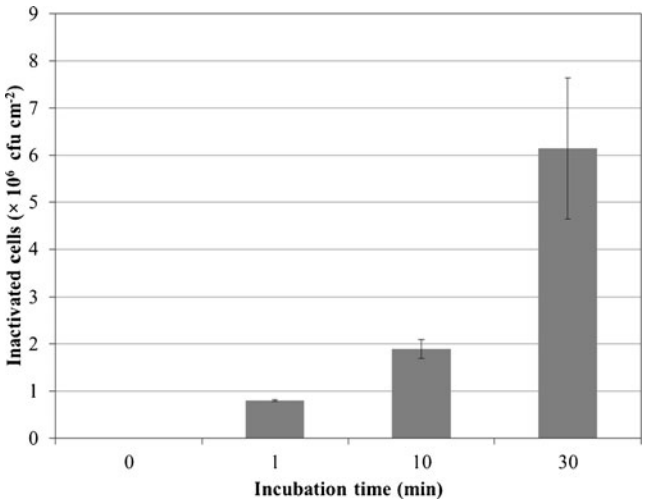

Fig. 3 Bactericidal efficiency of cicada wing surfaces against *P. aeruginosa* cells. The number of cells that were inactivated by the surface after 30 min reached 6.14×10^6 cfu cm^{-2}

Therefore, while the viability analysis performed here was based on the FDA guidelines, the inoculum size was increased to ensure that the results obtained were statistically significant. At the same time, care was taken not to increase the inoculum by such an amount that the bactericidal effect would not have been able to be detected by the plate count method.

Discussion

Until recently, antibacterial materials have relied one form or another of coating or impregnation with various antibacterial compounds, e.g., silver nanoparticles, polycationic compounds, etc. (Schierholz et al. 1998; Tiller et al. 2001; Shao and Zhao 2010). Such materials unfortunately have limited effective life spans, as leaching of the antibacterial chemicals will eventually lead to their concentration dropping below the bactericidal threshold (Schierholz et al. 1998). Surfaces such as the cicada wings presented here are not likely to suffer from the same limitations, as the mechanism of action is physical rather than chemical. The combined action of the bactericidal mechanisms and self-cleaning properties should ensure that the life span of the material is extensive.

Given that the wings are only effective against Gram-negative cells and show minimal activity against Gram-positive cells provides further evidence in support of the assertion made in the previous work on cicada wings that the bactericidal activity is mechanical in nature (Ivanova et al. 2012). Gram-positive cells have thicker layers of peptidoglycan and are therefore generally more rigid, which may explain their

increased resistance in comparison to Gram-negative cells. This is consistent with previous work in which the adsorption of bacterial cells onto the wing structures was mathematically modeled (Pogodin et al. 2012). It was suggested in this work that the primary factor determining the susceptibility of cells to the wing surface was the rigidity of the cells.

The knowledge presented here has the potential to be applied to several medical and industrial techniques. There are a number of fabrication techniques that show some promise for the replication of the nanopattern present on the surface of cicada wing nanopatterns (Zhang et al. 2006; Lim et al. 2009; Kostovski et al. 2010). The key limitations of these techniques that need to be overcome surround the resolution, on the nanometer scale, required to accurately reproduce these structures. There is, however, room for development of these techniques, which may allow achievement of the required resolution enhancement. Some examples are nanoimprint lithography (Zhang et al. 2006), self-assembly (Koch et al. 2008), femtosecond laser exposure (Kietzig et al. 2009; Fadeeva et al. 2011), and deep reactive ion etching (Choi and Kim 2006; Mischensko et al. 2010). Many surfaces fabricated by these techniques have demonstrated high potential for repelling bacterial cells (Díaz et al. 2007, 2009, 2010, 2011; Epstein et al. 2012; Hook et al. 2012), but thus far, none have been found to possess bactericidal activity. However, the bactericidal action of the nanopatterned cicada wings provides a sound basis for producing synthetic antibacterial surfaces.

In summary, the bactericidal activity of cicada wings was tested against seven bacterial species, covering every combination of cell morphology (rod-shaped and coccoid-shaped) and cell wall structure (Gram-positive vs Gram-negative bacteria). The wings exhibited effective and efficient bactericidal activity against Gram-negative cells, regardless of their morphology, while Gram-positive cells were found to be resistant to the antibacterial nature of the wing. The results of this work demonstrated that while the wing surfaces have little (if any) effect on the viability of Gram-positive cells, they are extremely effective in inactivating Gram-negative bacteria.

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